Recognition and manipulation of adult stem cells through haematopoietic and non-haematopoietic differentiation pathways for intended therapeutic clinical applications.

Olga Tura-Ceide

PhD
University of Edinburgh
2006

I declare that the work presented in this thesis is my own unless otherwise stated.

Olga Tura-Ceide
2006

Acknowledgements

I wish my very sincere thanks to all those without whom completion of this PhD would have been impossible. I would like to thank my supervisors Dr Robin Barclay and Dr Marc Turner for their advice and assistance. In particular I would like to thank Robin for all our fights, help and time spent. Laboratory colleagues have also played a major role in particular I would also like to thank Kay Samuel who has provided me excellent advice and for the times spent reading the manuscript.
Thanks must also go to Dr John Davies and Dr Huw Roddie for all their support from the beginning, collaborators Isabelle Heinisch, Nick Mills, Chris Millar and Ida Flisijn and everyone who has provided me many of the blood samples used in this thesis.
This study was supported in part by an educational grant from Chugai Pharma, UK and I wish to thank David Eves for his encouragement.
Finally I want to thank Jordi, without whose patience, love and encouragement none of this would have been achieved.

Abstract

In this thesis studies are presented which focus on autologous stem cells for autograft cellular therapies in (i) improvement of early haematopoietic reconstitution by *ex vivo* manipulation of graft cells, and (ii) assessment of the angiogenic potential of available clinical sources for development of therapies for vascular regeneration in ischaemic tissue.

The haematopoietic stem cell (HSC) is the best characterized of all adult tissue stem cells and conventionally HSCs from bone marrow or from G-CSF mobilised peripheral blood are reinfused to patients for haematopoietic restoration following myeloablation. In such procedures there remains a period following HSC autograft when patients are neutropenic and thrombocytopenic. A number of studies have used complex cytokine combinations to attempt simultaneous *ex vivo* expansion of progenitors of neutrophil and megakaryocyte lineages to reduce post transplant neutropenia and thrombocytopenia. Our results show that $CD34^+$ HSCs could expand into mature functional neutrophils under the influence of SCF+Flt3-L+G-CSF, but that the addition of other cytokines did not improve $CD34^+$ expansion, and the megakaryocytic growth factor TPO reduced neutrophil maturation.

Recent recognition that stem cells for tissues other than blood exist in the adult has stimulated clinical interest in their use for a range of regenerative therapies. Endothelial progenitor cells (EPCs) are stem cells with the potential to proliferate and differentiate into mature endothelial cells and to form blood vessels in a process resembling embryonic vasculogenesis. Autologous EPC transplantation may be used to promote endothelial reconstitution in patients with ischaemic or infarcted tissue. EPC appear to share many properties with haematopoietic stem cells (HSC), but while haematopoietic potential is now assessed by numbers of HSC expressing CD34, EPC determination remains ambiguous. Bone marrow and mobilised peripheral blood are HSC-rich sources which have been used for vasculogenic therapy. The studies presented here show that there is no correlation between the diverse EPC phenotype definitions used in the literature. There is no obvious relationship between the numbers of haematopoiesis-related $CD34^+$ or $CD133^+$ cells and outcome of the CFU-EPC assay. Further studies revealed that the cells responsible for these early outgrowth CFU-EPC colonies were $CD14^+$ plastic-adherent monocyte-like cells. Recent reports indicate that a peripheral blood monocyte-like population may also be considered as an EPC source with potential angiogenic clinical capability. Further work is required to better define the relative endothelial potential of these sources for development of clinical autograft vascular regenerative therapies.

Contents

Chapter 1

General Introduction

Introduction

The studies in this thesis focus on autologous stem cells for autograft cellular therapies, and are in two parts. The first part investigates possible improvement of early haematopoietic reconstitution by *ex vivo* manipulation of haematopoietic graft cells. The second part is an assessment of the angiogenic potential of available clinical autologous graft sources for development of therapies for vascular regeneration in ischaemic tissue. To place this in context and as an introduction, a brief history of clinical studies underpinning current concepts is presented below. However this is reinforced by major laboratory and animal studies which will be discussed in subsequent sections.

1.1 A brief history of the clinical background to regenerative cell therapy.

Current concepts of regenerative cell therapy have evolved principally from developments in blood transfusion, haematology, immunology and latterly embryology and developmental biology.

Advances in medicine have been driven by the continuing battle against disease. Throughout history people have been intrigued by the therapeutic possibilities of transplantation of organs and tissues from one body to another body, or from one part of the same body to another. During the fifteenth century we can find references in historical medical literature of attempted blood transfusions as well as the transplantation of teeth (presumably from cadavers). However, an understanding of the concept of blood circulation (1628 William Harvey) was critical to the development of blood transfusion. Initially, transfusion was attempted between dogs with some success (1665 Richard Lower). In the eighteenth century transfusions were done only sporadically, and were generally animal to human. Transfusion

was generally thought of as a cure for mental aberration or as a youth potion for the aged rather than as a treatment for blood loss. In 1818 James Blundell performed the first successful human blood transfusion for the treatment of postpartum haemorrhage. In 1901, Karl Landsteiner and colleagues discovered the ABO blood type system and subsequently in 1939 the Rh blood group system. Anticoagulant use was developed during World War I which allowed blood storage. Reports of organ transplantation, such as skin and corneal transplants appear in medical journals dating as far back as 1880. However, the first successful organ transplant was conducted by J Murray and colleagues in 1954 when they performed the first successful kidney transplant between identical twins. They also performed the first renal allograft in 1959 with the aid of immunosuppressive drugs. In 1958 Jean Dausset described the first of many human histocompatibility antigens. Heart, liver, and pancreas transplantation and the first successful bone marrow transplantation followed soon after, and have progressed with better understanding of histocompatibility and development of immunosuppressants.

In the 1970's a large number of allogeneic bone marrow transplantation procedures were performed using HLA-matched sibling donors for patients with end-stage leukaemia or aplastic anaemia, as reviewed by Chao and Blume, 1989. Bone marrow transplantation soon became the definitive treatment for immune-deficiency diseases, blood disorders, and numerous other indications, as reviewed by one of the pioneers, (Good, 2002). These treatments rely on the patient lacking an immune system as in some congenital immunodeficiencies, or on endogenous bone marrow destruction through myeloablation and immunosuppression to prevent or reduce graft rejection, but the patients are at risk of graft versus host disease. Patients also suffer from a period of neutropenia and thrombocytopenia during which they are at risk from infection and bleeding disorders. Use of autologous bone marrow, which developed from the success of allogeneic bone marrow transplants, obviates the need for histocompatible donors or immunosuppression, and avoids graft-versus-host disease, but may lack a graft-versus-tumour effect in oncology cases and the graft should be free of residual tumour. The main benefit of autologous bone marrow is to set aside restorative amounts of a patient's haematopoietic cells which can reconstitute the patient following intense or cumulative therapies which have marrow toxicity (reviewed by Chao and Blume, 1990). Later, the use of autologous peripheral blood stem cells was found to reduce the period of neutropenia and thrombocytopenia. Today, while not applicable in all cases or diseases, autologous peripheral blood, potentiated by mobilisation of haematopoietic cells from bone marrow by recombinant cytokines, is the most common source of haematopoietic

stem cells for transplant. Nevertheless, the period of neutropenia and thrombocytopenia still remains clinically significant, and investigation of a means to reduce this neutropenia is the topic of the first part of this thesis.

Organ transplantation has come a long way since the pioneering clinical procedures of the 1950's and 1960's, with advances in histocompatibility assessment, surgical techniques and development of more effective drugs to prevent organ rejection which have enhanced graft preservation and allograft survival. However, the number of patients awaiting organ transplant is large and growing. It was hoped that organ preservation and xenotransplantation (the transplantation into people of organs from other species) would provide a ready supply of organs, but immunologic difficulties and concerns regarding transmission of zoonotic diseases currently limit their use.

It may be that the solution to the shortage of organs for transplantation lies in stem cell research and tissue engineering. During embryonic development pluripotent stem cells divide and differentiate to form all the specialised somatic cells of the body of which the various tissues and organs are composed. If such pluripotent stem cells could be harvested and encouraged to differentiate into almost any type of cell needed in the body then this might lead to cellular therapies for organ damage. This could include new nerve cells for people with brain disorders or spinal injuries, new heart muscle cells to repair damaged hearts and new and effective treatment for strokes, burns and arthritis. Recent advances in embryology and developmental biology have resulted in isolation and manipulation of pluripotent embryonic stem cells (ES) whose clinical potential has excited much research and controversy. While this potential advances towards clinical realisation, embryonic stem cells currently suffer from two major clinical barriers. Firstly the concern that they may escape growth limitation and regulation to generate teratoma-like tumours, and secondly that they will be rejected by their recipients due to histocompatibility differences since, unlike the recipients of haematopoietic stem cells, the patients who might benefit from ES therapies are unlikely to be congenitally or therapeutically immunocompromised or to be justifiably considered for chronic immunosuppression.

It has been recognised for some time that in adults certain tissues such as blood, skin, hair, and organs such as liver, can extensively renew and regenerate, presumably from stem cells specific for those tissues. Indeed therapies such as haematopoietic reconstitution discussed above are predicated on the existence of multipotent haematopoietic stem cells capable of generating the different mature cellular components of blood. Pursuit of the fundamental adult multipotent haematopoietic stem cell, whose definition still remains elusive, has been a

contributing impetus which has led in recent years to a recognition that in adults stem cells may participate in repair and regeneration of a variety of tissues and organs whose constituent somatic cells were previously thought to rarely, if ever, regenerate themselves. One recent controversial idea is that adult stem cells have the potential for transdifferentiation; in other words, that they are able to transmutate from one type of tissue cell to another, so that a haematopoietic stem cell might be capable of generating other tissue such as, for example, muscle or nerve. Other recent ideas are that different tissue specific stem cells of restricted potency reside within specialised stem cell niches in different tissues; fundamental adult stem cells exist which resemble embryonic stem cells in their pluripotency; novel cells are incorporated into tissue by fusion between recognised stem cells such as haematopoietic stem cells and resident tissue somatic cells; or that extraneous cells contribute to tissue regeneration by paracrine influences on resident cells through cytokine secretion. Whether some or all of these processes operate in the adult to some extent remains to be confirmed conclusively, but it is becoming evident that there is a potential for regenerative therapies based on adult stem cells beyond the established haematopoietic therapies.

Therapeutic use of adult stem cells may solve the concerns relating to embryonic stem cell potential for teratoma formation, but would still leave the problem of tissue histocompatibility necessitating chronic immunosuppression, if allogeneic donor stem cells were employed. However, none of these problems arise with the use of autologous adult stem cells, which have the most immediate prospect of early therapy development and implementation.

Currently, methods for expanding and specifically differentiating pluripotent stem cells are the subject of intensive research. Certain adult stem cell lineage relationships are emerging that appear promising for therapeutic exploitation. Prominent among these are mesenchymal stem cells for bone and cartilage regeneration and the haemangioblast, a common precursor of haematopoiesis and vasculogenesis that persists in the adult. In the latter context, a number of recent clinical studies have employed autologous sources of haematopoietic stem cells to elicit apparent revascularisation of ischaemic tissues, backed up by a number of experimental laboratory studies. However, there is no consistent definition of which cells are active in this revascularisation, nor any means of assessing the qualitative or quantitative vasculogenic potential of any autograft source for such therapy or its response to any intended *ex vivo* manipulation or enhancement of clinical vasculogenic potential. The second part of this thesis represents an attempt to systematise and reconcile some of the various and conflicting definitions of endothelial

progenitor cells (EPC) held to be responsible for vasculogenesis in the adult, to better assess the available sources of autologous EPC which might be proposed for vascular regenerative therapy of ischaemic heart disease and critical limb ischaemia.

1.2 What is a Stem Cell?

A stem cell is defined as a cell with the unique ability to self-replicate for indefinite periods of time (maybe thorough the entire life of the organism), and which under the appropriate signals can give rise (differentiate) to many different cell types that make up the organism and with the potential for *in vivo* reconstitution of the given tissue upon transplantation. Stem cells have the potential to develop into mature cells that have characteristic morphology and specialized functions, such as heart cells, bone cells, skin cells, nerve cells or blood cells (http://www.nih.gov/news/stemcell/scireport.htm). Stem cells have the distinctive property, by comparison to the large majority of cells of the body, to be uncommitted and remain as such until they receive a signal to generate specialised cells (Lemoli *et al.*, 2005). The potential degree of cellular differentiation varies among stem cell populations. A fertilised egg is a *Totipotent* stem cell that can produce cells that arise from all three germ layers (endoderm, mesoderm and ectoderm) and are able to form extraembryonic tissues such as the supporting trophoblast required for the survival of the developing embryo. *Pluripotent* stem cells can produce cells that arise from all three germ layers and germ cells but not extra-embryonic tissue. Pluripotent stem cells such as embryonic stem cells (ES) and embryonic germ cells (EG) are isolated from the inner cell mass of the blastocyst or from primordial germ cells of an early embryo respectively (Lakshmipathy and Verfaillie, 2005). Unlike differentiated somatic cells, ES cells do not require any external stimulus to initiate DNA replication and spend most of their time in the S-phase of cell cycle. *Multipotent* stem cells give rise to all tissues derived from one of these germ layers. *Tissue-restricted* multipotent stem cells or *oligopotent* stem cells give rise to lineage-restricted tissue specific cell types and *Unipotent* stem cells can only generate one cell type and may be better defined considering the stem cell definition, as committed progenitor cells (Marshak *et al.*, 2001). Different groups have used different terminologies to define stem cell differentiation potential. In this study, the classification described above is used until a final consensus definition is reached (See also Figure 1.1).

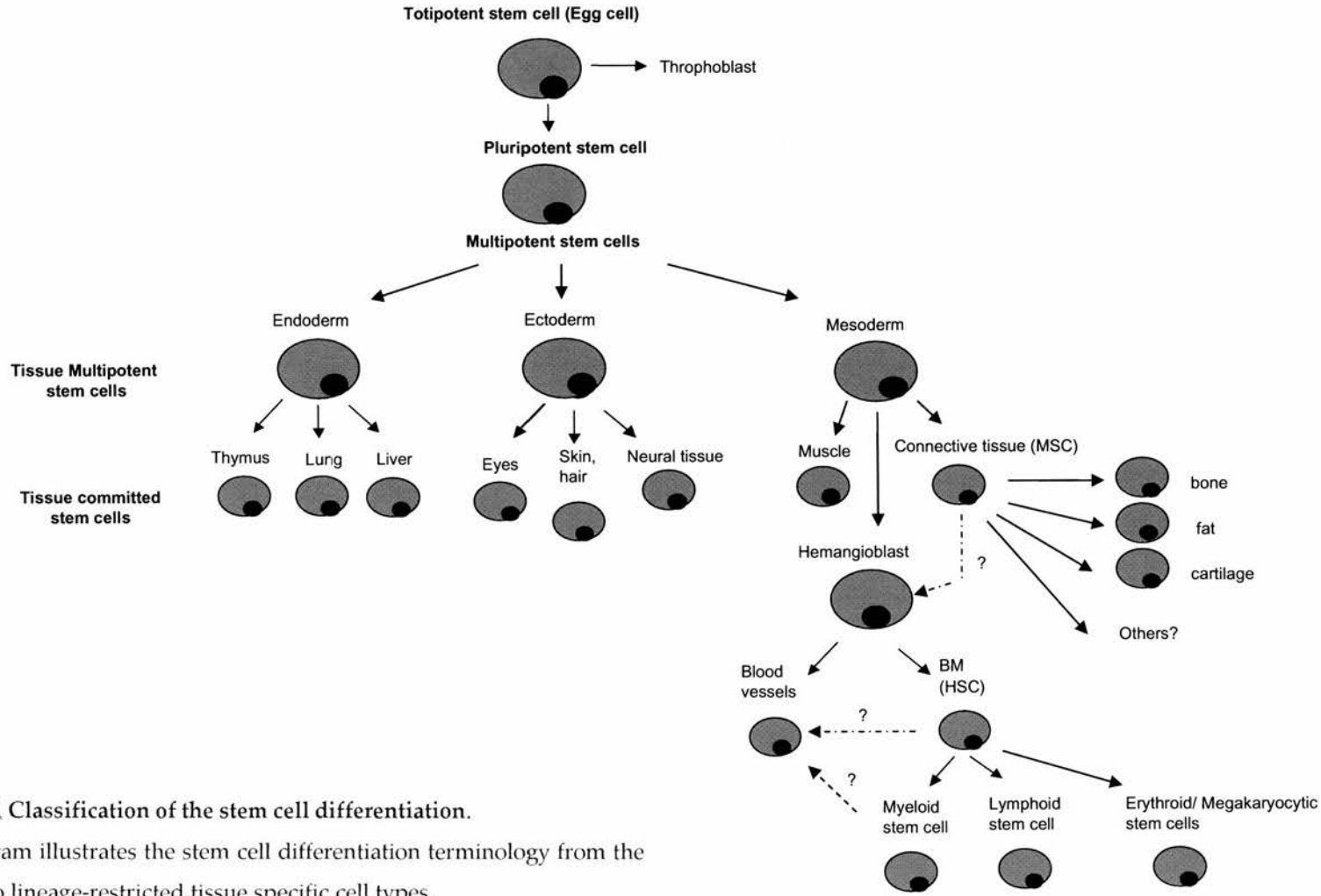


Figure 1.1 Classification of the stem cell differentiation.

The diagram illustrates the stem cell differentiation terminology from the egg cell to lineage-restricted tissue specific cell types.

1.2.1. The human embryonic stem cell

Embryonic stem cells (ES cells) are pluripotent stem cells that can be grown *in vitro* indefinitely in their undifferentiated state, undergoing an unlimited number of symmetrical divisions, whilst maintaining a normal stable diploid karyotype (long-term self-renewal). A single ES cell can also give rise to differentiated cell types that are representative of all three primary germ layers of the embryo (endoderm, mesoderm, ectoderm) (Amit *et al.*, 2000; Laslett *et al.*, 2003). An ES cell is defined by its origin, the blastocyst, one of the earliest stages of embryonic development before implantation in the uterine wall (http://www.nih.gov/news/stemcell/scireport.htm). The ES cells specifically come from the inner cell mass of the blastocyst. These cells proliferate extensively in the embryo, and they are capable of differentiating into all types of cells that occur in the adult both *in vitro* and *in vivo*. (Pera *et al.*, 2003; Reubinoff *et al.*, 2000; Thomson *et al.*, 1998). Human ES cells have been shown to differentiate *in vitro* into a wide variety of cells including mature neurons, astrocytes, oligodendrocytes, cardiomyocytes, endothelial cells, insulin-producing cells, hepatocyte-like cells and haematopoietic colony-forming cells. (Reviews; Verfaille a) *et al.*, 2002; Passier and Mummery, 2003). Therefore, these cells have potential for use in stem cell transplantation, gene therapy and tissue regeneration applications (Passier and Mummery, 2003). Transplantation of human derived-ES cells could replace or restore tissue which has been damaged by disease or injury and be used to treat diseases such as Parkinson's disease, diabetes or heart failure. Human blastocysts for the establishment of renewable hES cell lines could be obtained from either supernumerary embryos or from embryos specifically produced for research purposes. One of the advantages of using ES cells as compared to adult stem cells is that ES cells have an unlimited ability to proliferate *in vitro* and are more likely to be able to differentiate into a broad range of cell types. Despite their vast potential, non trivial concerns such as ethical issues about the moral rights of the embryo, scientific debates concerning teratoma formation, and histocompatibility issues are delaying immediate clinical application of ES cells.

1.2.2. The adult stem cell

Stem cells are also present in adult life and by contrast with ES cells their origin is not well known. Adult stem cells are traditionally defined as undifferentiated, rare, difficult to identify cells found in many differentiated tissues which can renew themselves and with certain limitations, give rise to all the specialized cell types of the tissue from which they originate (http://www.nih.gov/news/stemcell/

scireport.htm). The main function of adult stem cells is to maintain homeostasis of their stem cell compartment during the entire lifetime of the organism and with limitation replace dying cells caused by injury or disease (Lemoli *et al.*, 2005). Most adult tissues are capable of some degree of regeneration and this function might be attributed to the adult stem cell population. The definition of an adult stem cell is that upon transplantation it should repopulate the tissue of origin, integrate into the tissue and give rise to fully differentiated functional cells (Lemoli *et al.*, 2005). The list of adult tissues reported to contain stem cells is growing and includes bone marrow, peripheral blood, spinal cord, dental pulp, blood vessels, skeletal muscle, epithelia of the skin and digestive system, cornea, retina, liver, pancreas, brain and heart (the last two organs were previously considered to have minimal or no cell turnover in adult life) (http://www.nih.gov/news/stemcell/scireport.htm). These stem cells are more abundant in tissues with a high renewal rate, such as blood or epithelia and less frequent in tissues or organs with less renewal capacity such as myocardial muscle or the central nervous system (Lemoli *et al.*, 2005). However, the extent to which stem cells actually participate in organ maintenance and repair in these low-proliferative tissues such as heart or brain it is under discussion. It is also not known whether adult stem cells are retained from the embryonic stage or they arise genuinely in adult life.

Adult stem cells are thought to be in a quiescent state, where the *in vivo* environment (also called the stem cell niche) plays a very important role in balancing their cellular needs until they are activated by disease or tissue injury. The factors that stimulate stem cells to become active and relocate to sites of injury or damage are also unknown.

As defined above, adult stem cell function was assumed previously to be restricted to cell lineages present in the organ from which they were derived and to be involved only in their maintenance and repair (Passier and Mummery, 2003). However, increasing evidence suggest that some adult stem cells under appropriate culture conditions may be more pluripotent cells, switching cell fate, capable of crossing tissue-lineage boundaries and producing cell types other than the cell type in the tissue of origin (Krause *et al.*, 2001; Martin-Rendon and Watt, 2003). These properties have been termed adult stem cell plasticity. The discovery of adult stem cells in many more tissues than previously thought provides new opportunities in stem cell biology for novel therapeutic possibilities (Krause *et al.*, 2001).

1.2.2.1 Adult stem cell plasticity

Adult stem cells are an attractive source of cells for therapy, especially in view of the recent claims that they are remarkably plastic in their developmental potential when exposed to new environments. Proponents of plasticity maintain that neurons, skeletal, epithelial, cardiac myocytes, hepatocytes, endothelial cells and bone cells can all be derived from primitive pluripotent bone marrow cells. If stem cell plasticity is real, we are challenged to reconsider the validity of long-held dogma regarding stem cell single lineage specificity in adults. However, reports of stem cell plasticity have caused both excitement and scepticism because some of these claims have been either difficult to reproduce or shown to be misinterpretations, leaving the phenomenon of adult stem cell plasticity unclear (Lakshmipathy and Verfaillie, 2005). Plasticity has to be defined as the capacity of a single cell to differentiate into multiple cell lineages functionally *in vitro* and *in vivo* and the engrafted tissue has to persist after cell transplantation (Lakshmipathy and Verfaillie, 2005). There are at least four different ways which plasticity can be explained: Stem cell pluripotency; transdifferentiation; cell fusion or multipotency heterogeneity (Martin-Rendon and Watt, 2003).

Krause *et al.*, (2001) published that a single mouse bone marrow-derived stem cell transplanted into lethally irradiated mice not only resulted in long-term reconstitution of the haematopoietic system but also differentiated into mature epithelial cells with a non-haematopoietic potential. Thus, they isolated a pluripotent lineage-negative bone marrow-derived cell able to differentiate into haematopoietic and non-haematopoietic cells. Also Theise *et al.* a), (2000) reported that injection of unseparated mouse bone marrow-derived cells resulted in localisation of some of these cells into the liver and their differentiation into hepatocytes. Similar results were seen in humans (Theise *et al.* b), 2000). Thus, in both humans and in the experimental animals, engraftment by bone marrow cells as hepatocytes could be seen in the absence of severe injury (Theise *et al.* a) 2000; Theise *et al.* b), 2000). Mezey *et al.*, (2003) showed the presence of donor cells in human brains after bone marrow transplantation for lymphoid leukaemias or immune deficiencies. Moreover, murine and human neural stem cells have been reported to be able to generate myoblasts *in vitro* (Tsai and McKay, 2000) and when transplanted into muscle (Galli *et al.*, 2000). Unfortunately, up to now, most of the studies examining the phenomenon of stem cell plasticity by pluripotency have not definitively shown that significant levels of unexpected multiple lineage differentiation are derived from the same single cell and that these differentiated cells could function *in vitro* and *in vivo*.

There is other evidence of the existence of a pluripotent stem cell in the adult (Lakshmipathy and Verfaillie, 2005). Multipotent adult progenitor cell (MAPC) seem to be capable of differentiating into many different tissues other than the tissue of origin. Jiang *et al.*, (2002) suggested that MAPC derived from bone marrow, brain or muscle may be related to ES cells. Mouse MAPC like mouse ES cells required leukaemia inhibitory factor (LIF) to be expanded whereas human MAPC, like human ES cells, do not require it. Moreover MAPC express transcriptions factors such as Rex1 and Oct4 which are important in maintaining ES cells undifferentiated (Jiang *et al.*, 2002). In addition, like ES cells, mouse bone marrow-derived MAPC contributed to all somatic cell types when injected in a mouse blastocyst. However, these cells only showed this potential after *in vitro* culture. Thus, further studies need to demonstrate that such cells exist *in vivo*. Whether these cells eventually are the same as the bone marrow lineage-negative cells enriched by Krause *et al.*, (2001) is not known. Better MAPC phenotypic characterisation is needed.

Fernandes *et al.*, (2004) identified a cell type derived from the skin (SKP) with potential that is not restricted to skin. The assumption that each tissue contains a specific stem cell subtype committed to tissue-restricted differentiation may not be complete (Zipori, 2005).

Another explanation of stem cell plasticity would be transdifferentiation. This process considers the ability of a lineage-committed stem cell from a specific tissue to differentiate into cell types of another tissue. In this, the nucleus of the transplanted cell would have to undergo reprogramming and up-regulate genes and proteins consistent with the new lineage. There is evidence in nature that such a phenomenon can occur (Lakshmipathy and Verfaillie, 2005); e.g. in young newts in which a limb is removed, cells in the blastema undergo de-differentiation and then re-differentiate to make most of the cell types of the regenerating limb (Brockes, 1997). Whether such a phenomenon occurs in humans and if it does whether adult stem cells normally exhibit transdifferentiation or they only transdifferentiate when manipulated in the laboratory also remains unknown.

Recent findings also challenged the nature of the stem cell plasticity by pluripotency or transdifferentiation when cells were shown to be able to express different phenotypes by cell-cell fusions (Martin-Rendon and Watt, 2003). Fusion between bone marrow and liver cells generated hepatocytes in mice (Vassilopoulos *et al.*, 2003; Wang *et al.*, 2003). Alternatively, plasticity could be explained by heterogeneity. Bone marrow could be considered a reservoir of a mixture of different lineage-committed stem cells with different plastic potential capacities. Some of these cells could still retain a high pluripotency capacity while others, as they mature, lose this

plasticity as genes are switched off at each step of differentiation. Most of the experiments studying stem cell plasticity have used non-purified populations and even where haematopoietic stem cells were enriched, the recovered population was still heterogeneous and may still have contained some pluripotent non-HSC cells which could differentiate into cells of non-haematopoietic lineage. Thus, we could accidentally interpret that HSC were responsible for the non-haematopoietic cell development, for example. Whether pluripotent plastic stem cells are somehow retained into adult postnatal life from fetal development, restrained from differentiating as proposed by Ratajczak *et al.*, (2004), is not known. Ratajczak *et al.* hypothesized that pluripotent stem cells circulate at a low level in the peripheral blood under normal steady-state conditions, maintaining a pool of stem cells in peripheral tissues, and their levels increase in peripheral blood during stress/tissue injury. The nature of these highly pluripotent cells are not known and the lack of definitive markers makes their identification even more difficult.

It is still unknown if pluripotency, transdifferentiation, cell fusion or heterogeneity makes a progenitor/stem cell or a more mature cell change, and maybe all coexist in the stem cell world. Research is continuing and many questions remain to be answered. To verify or refute the various hypotheses requires careful experimentation, but the potential clinical applications of adult stem cells in transplantation are vast.

1.2.2.2 Adult stem cell therapy

Regenerative medicine and cell therapy have become a major focus of modern therapeutic approaches for the treatment of a wide variety of different inborn, acquired and degenerative diseases, otherwise untreatable conditions. Due to histocompatibility issues, concerns about teratoma formation, and ethical considerations, hES cells are at the moment restricted to experimental *in vitro* studies and their therapeutic potential remains to be determined. In contrast, adult human stem cells, with their newly documented plasticity, could be a more immediate potential source for therapeutic application. However, more convincing experiments are required with better adult stem cell characterisation (Conrad and Huss, 2005). There is also a requirement for development of robust, safe and standardized strategies for handling and preparing the cells for transplantation.

Adult haematopoietic stem cell transplantation has been extensively used clinically for several decades for haematopoietic reconstitution after myeloablative chemotherapy or in congenital immunodeficiencies. Using the haematopoietic system as a model, this could be extended and adult stem cells might also be

transplanted to restore other damaged tissues such as blood vessels, bone, or more speculatively liver, myocardium or brain. Adult haematopoietic stem cells have been used clinically across histocompatibility barriers from allogeneic sources because their recipients had their immune system largely destroyed by myeloablative chemotherapy. Graft versus host disease (GvHD) is also a significant clinical problem, making autologous HSC sources the preferred option in many instances of stem cell transplant recipients for bone regeneration or vasculogenesis which are not likely to receive myeloablation. In addition to the more favourable treatment related toxicity profile of autologous transplantation, this approach eliminates the risks of the recipients acquiring untoward infection from the donors and has none of the ethical problems currently associated with the use of ES cells.

1.2.2.3 Haematopoietic adult stem cells

As described above, there are many different adult stem cells. The haematopoietic stem cell (HSC) is the best characterized of all adult tissue stem cells. Mesoderm derived, multipotent, rare (less than 1/1,000 nucleated bone marrow cells), haematopoietic stem cells are considered capable of both self-renewal and differentiation into any cell of the mature haematopoietic lineage (erythrocytes, neutrophils, monocytes, B lymphocytes, T lymphocytes, NK cells, eosinophils, basophiles and megakaryocytes) under the control of transcription factors. Although these mature cells have distinct morphology and function they are derived from a common multipotent stem cell pool (Forbes *et al.*, 2002; Orkin, 2000). HSCs can be isolated from bone marrow, foetal liver, umbilical cord blood and although normally rare in peripheral blood they can be enriched there by stimulation and release from the bone marrow to peripheral blood by G-CSF administration. Haematopoietic reconstitution has been proven *in vivo* by transplantation of single stem cells into syngeneic animals (Krause *et al.*, 2001; Osawa *et al.*, 1996). Despite the ability to culture haematopoietic progenitor cells to particular committed lineages, a reliable method for *in vitro* culture of self-renewing stem cells which maintains them in an undifferentiated state has not been achieved as yet for human HSC (Uher *et al.*, 2003). The intrinsic and extrinsic signals that regulate haematopoietic cell fate are not yet fully understood.

Significally, some recent studies referring to stem cell plasticity indicate that HSC also have the potential to generate cell types other than haematopoietic cells such as bone, cartilage, neural cells, muscle, skin, blood vessel endothelia, epithelial cells, hepatocytes (Orkin and Zon, 2002; Moore, 2002. Theise *et al.* showed that blood and bone marrow stem cells can generate hepatocytes and vice-versa (Theise *et al.*

a) 2000; Theise *et al.* b)). Similar results have been found in the brain following human bone marrow transplants (Mezey *et al.*, 2003). Once more whether it is due to haematopoietic stem cell pluripotency, transdifferentiation, multipotency-heterogeneity, or due to the existence among the enriched HSC of remaining pre-HSC pluripotent stem cells that have still the potential to become non-haematopoietic stem cells is still not resolved.

1.2.2.4 Non-Haematopoietic adult stem cells

Non-haematopoietic adult stem cells are adult stem cells capable of developing into mature cells of tissues other than blood. Endothelial progenitor stem cells, skeletal muscle stem cells, epithelial cell precursors in the skin and digestive system and stem cells in the pancreas and liver have recently been found.

Mesenchymal stem cells (MSCs) are one kind of non-haematopoietic stem cell found in bone marrow (Conget and Minguell, 1999), adipose tissue (Zuk *et al.*, 2002) and other tissues such as fetal liver, peripheral blood, lung and cord blood (reviewed in Le Blanc and Pittenger, 2005). These cells are defined by their lack of haematopoietic markers (CD45-negative), their expression of certain mesenchymal markers after culture, their tendency to adhere to plastic and their ability to differentiate into adipocytes, osteocytes or chondrocytes. More controversially, it has been shown that they may also generate a wide variety of other cell types including skeletal muscle, neuronal cells, hepatocytes and vascular endothelial cells under the correct conditions (Le Blanc and Pittenger, 2005). MSC have already been used clinically for the treatment of bone disorders such as osteogenesis imperfecta (Le Blanc and Pittenger, 2005) and could be a potential useful source to treat bone, cartilage or tendon diseases (Lakshmipathy and Verfaillie, 2005). Finally, others have shown that a rare cell type, isolated from bone marrow and other tissues such as brain and muscle, termed multipotent adult progenitor cell (MAPC) can be expanded with a high proliferative rate and after culture show potential to differentiate into endothelial, endodermal and neuronal lineages *in vitro* and *in vivo* (Reyes and Verfaillie, 2001; Jiang *et al.*, 2002, Muguruma *et al.*, 2003). MAPC have many similarities to MSC, which are also present in bone marrow, but their relationship to MSC and whether MAPC exist *in vivo*, or are only induced during an extensive *in vitro* culture period, is still unknown. (Lakshmipathy and Verfaillie, 2005) (See also 5.1.10).

1.2.2.5 Identification parameters of the human haematopoietic stem cell

Attempts to isolate HSCs in early studies were developed by determining physical parameters such as size, buoyant density, and electrical charge characteristics. Before the widespread availability of flow cytometry for analysis, polyclonal antibodies, colony assays, immunoreactive columns and immunomagnetic beads were used to isolate and characterise haematopoietic cells (Metcalf et al., 1971). In 1980s, the characterisation of human HSC progressed due to the use of the flow cytometry and the advent of monoclonal antibody (MAb) technology. Many studies have been made to identify the surface phenotype of HSCs. Availability of highly enriched populations of stem cells should facilitate the research in haematopoiesis and clinical stem cell transplantation. During the last two decades, investigators identified a number of surface molecules to define a HSC. For more than a decade CD34 has been the best-known marker of HSCs. In (1988) Berenson *et al.* using anti-human CD34 antibody clone 12.8, reported successful haematopoietic reconstitution in baboons with selected $CD34^+$ bone marrow cells. The discovery of CD34 accelerated the development of protocols for the purification of human HSCs (Andrews *et al.*, 1986). It appears that there is a progressive decline in CD34 antigen expression along with haematopoietic cell differentiation at later stages of haematopoiesis. The CD34 molecule is a 115-KDa type 1 integral sialomucin membrane protein whose function has proven enigmatic (Lanza *et al.*, 2001). This antigen is highly glycosylated, and differential susceptibility to enzymatic cleavage, tissue reactivity and western blotting analysis have established **a** classification of the various epitopes of the CD34 antigen. See table below (Lanza *et al.*, 2001).

EPITOPE CLASS	CLONES	CD34 REACTIVITY		
		paraffin section	frozen section	western blotting
Ia. Highly sensitive to neuraminidase, and glycoprotease	12.8, BI.3C5, Immu409, B-G25, B-H21	Positive	Positive	Most positive
Ib. Partially sensitive to neuraminidase, sensitive to glycoprotease	My10, ICH3, Immu133, 14G3, ICO-115	Positive	Positive	Most positive
II. Resistant to neuraminidase, sensitive to glycoprotease	QBEnd10, 43A1, MD34.3, MD34.1*, MD34.2, 4A1, 9044, 9049*, 45.28	Positive	Positive	Positive
III. Resistant to neuraminidase and glycoprotease	TUK3, 115.2, 8G12, CD34-9F2, 581, 553, 563, Birma-K3, BF23, 6A6, 7E10, 4H11	Negative	Positive	Some weak positive

Neuraminidase from *Vibrio cholera*.
Glycoprotease from *Pasteurella haemolytica*.
*Although classified as class II reagents in the Vth Workshop, some participants found these MAbs to be partially sensitive to neuraminidase using both serology and immunoblotting procedures, and assigned them to class Ib (see text).
Chymopapain also cleaves class I and class II epitopes but in the hands of some Workshop participants, also cleaves class III epitopes (see text).

The AC136 clone which recognizes a class III epitope of the CD34 antigen was used for the experiments presented in this thesis. This monoclonal antibody clone recognises a different epitope from the QBEND/10 clone used for MACS® Microbeads (Miltenyi Biotech) and is therefore suitable for evaluatation of MACS® Separations.

Currently, adult haematopoietic stem cells are routinely isolated from the bone marrow, umbilical cord and peripheral blood based on the expression of the CD34 antigen, however their isolation at sufficient purity and quantity remains difficult. Controversy arose when different investigators found a CD34-negative-lineage-negative-c-kit$^+$ population that could produce a long-term lympho-haematopoietic reconstitution of lethally irradiated mice (Osawa *et al.*, 1996). Morel *et al.*, (1996); Morel *et al.*, (1998) and Donnelly *et al.*, (1999) have shown that in mice stem cells are present in both CD34-positive and CD34-negative populations of the bone marrow. Moreover, reports from Goodell *et al.* (1996); Goodell *et* al., (1997) observed that side population (SP) cells, which are a fraction of stem cells defined by their side scatter characteristics and exclusion of Hoechst dyes as measured by flow cytometry, are highly enriched for haematopoietic stem cells which are CD34-negative or low. Hence, such HSCs express the Bcrp1 (also known as ABCG2) transporter, which effluxes certain molecules including Hoechst 33342, a supravital DNA stain. These low fluorescence-expressing cells are highly enriched for HSCs. Human HSCs also express CD133 surface antigen (prominin-1) (Yin *et al.*, 1997), a 120KDa five transmembrane domain glycoprotein (5-TM) found originally on neuroepithelial stem cells in mice, and expressed on primitive cell populations such as some CD34 haematopoietic stem cells, neural and endothelial cells. CD133$^+$ neurosphere cells differentiate into both neurons and glial cells (Uchida *et al.*, 2000). The CD133 positive fraction of human bone marrow, cord blood and peripheral blood has been shown to efficiently engraft in xenotransplantation models (de Wynter *et al.*, 1998). No natural ligand has yet been demonstrated for the CD133 molecule, and, like CD34, its function in haematopoietic tissue is unknown. Anti-CD133 antibody provides an alternative to the widely used anti-CD34 antibody for the selection and characterisation of cells necessary for both short- and long-term engraftment in transplant situations and for studies of *ex vivo* expansion strategies, and for gene therapy (Yin *et al.*, 1997). Furthermore, Quesenberry *et al.* (2002) suggested that HSCs are continuously altering their phenotype and these alternations are reversible. Thus, many described HSC phenotypes could represent a single cell in different functional states. The wide variety of different marrow-derived haematopoietic stem cells may reflect a hierarchical stem cell system of extraordina-

ry complexity in which phenotypes and functional status may overlap. Lemoli *et al.*, (2005) suggested that rather than a hierarchical transition from stem cell to progenitor cell, fluctuations continue to exist in which stem cells adjust their phenotype depending on the cell kinetic state, activated by their genetic program or microenvironmental stimuli. Moreover, Zipori, (2005) suggested the concept of "stem state" where stemness and plasticity are a state rather than cellular entity. These studies have emphasised the heterogeneity of HSCs in terms of proliferative and self-renewal capacities. Although these recent studies indicate that CD34 is not the only marker of all human HSCs (Bonnet, 2002) and is also found on mature endothelial cells ,it is still, almost universally, the most commonly used marker for identification of HSC and for assessing the potency of clinical autograft and allograft cell harvests for haematopoietic reconstitution (Sutherland *et al.*, 1996).

Chapter 2:

Materials and methods

2.1 Cell sources and sampling

2.1.1 General sample collection procedures

All cells were obtained from samples from human sources. In all cases, appropriate ethical committee consent and institutional procedures involving written informed consent from each patient were applied. Ethical committee approval was obtained (LREC/1993/4/76 – Lothian NHS Board) for sampling haematopoietic stem cell sources (bone marrow, mobilised peripheral blood, umbilical cord blood) and normal peripheral blood for ongoing studies in SNBTS R&D. These were originally granted to Dr ML Turner. These were extended as necessary by Drs Turner and Barclay. The most recent approved extension was granted for follow-up samples following G-CSF administration (25th August 2005).

Peripheral blood cells were obtained from venous blood samples collected in heparin unless otherwise stated or where it is stated that buffy-coat leucocytes were used, where buffy coats were obtained in blood bags from the leucocyte-rich layer at the sedimented red-cell/plasma interface following routine preparation of platelets from fresh blood donations to the Scottish National Blood Transfusion Service. Umbilical cord blood was aspirated from the umbilical placental veins following normal elective caesarean delivery and collected into heparinised 50 ml containers. Bone marrow samples (3ml) were obtained in EDTA 5ml tubes by aspiration from the posterior iliac crest of haematologically normal donors sampled for routine clinical investigations of non-malignant diseases: residual fresh sample was made available for these studies. Peripheral blood samples rich in haematopoietic stem cells (HSC) following mobilisation of HSC by granulocyte colony stimulating factor (G-CSF) were termed "mobilised peripheral blood" (mPB), and were venous blood samples

(10ml) collected in heparin from donors immediately following cell-separator leukapheresis collection of G-CSF mobilised peripheral blood stem cells (PBSC) for transplant. In most cases these were adult patients donating for autologous PBSC transplant during disease remission; in some cases donors were healthy adult volunteers (usually patient relatives) donating for allogeneic PBSC transplant. Patient samples of mPB were drawn from patients with the following underlying diseases: Myeloma, Hodgkins and non-Hodgkins lymphoma, Chronic lymphocytic leukaemia (CLL) and chronic myeloid leukaemia (CML).

In clinical studies 40 patients undergoing elective coronary angiography participated in this study. All the patients were recruited from the Medical Day Case Unit at the Royal Infirmary Edinburgh following referral for diagnostic angiography to investigate symptoms suggestive of stable angina. Patients with a recent acute coronary syndrome or coronary intervention (<3 months), renal or hepatic failure, or a systemic inflammatory disorder or malignancy were excluded from the study. 20 patients underwent diagnostic coronary angiography alone, and 20 required balloon angioplasty and stenting because of flow limiting coronary stenosis. A venous cannula (17-gauge) was inserted into a large subcutaneous vein of the ante-cubital fossae for blood sampling before, immediately after and at 6 and 24 hours following angiography. EDTA anti-coagulated blood (Sarstedt-Monovette, Germany) was collected for flow cytometry, real-time PCR and for preparation of plasma for storage.

For other clinical studies 14 patients, who were undergoing elective abdominal aortic aneurysm repair, were used. 5ml of blood was taken in EDTA coated tubes pre-operative and at the following times post-operatively: 24 hours, 48 hours and after 5 days following operation. Moreover 5ml of EDTA anti-coagulated blood samples were collected from 45 patients with type I diabetes and from 34 controls subjects (control group).

Full blood and differential counts using an autoanalyzer (Sysmex UK), including monocyte counts, were obtained from each patient, and at each time point.

2.1.2 Collection of samples for study of platelet-leucocyte aggregation

Platelets are able to adhere to leucocytes, forming Platelet Leucocyte Aggregates (PLAs). Some endothelial markers such as vWf and PECAM (CD31) are also expressed by the platelets, therefore elimination of the platelets bound to leucocytes in the samples was considered necessary. A comparison between venous blood samples collected into Heparin, EDTA or Citrate anticoagulant was carried out. Also a heparinised sample was divided in different tubes and each tube was washed

individually with EDTA/PBS (0.1%), Trypsin/EDTA (TVP), Chloroquin (2mM), Lignocaine (2mM), Chloroquin/EDTA (2mM/0.1%) and Lignocain/EDTA (2mM/0.1%) *(see reagent list)*.

2.1.3 Endothelial cell lines

2.1.3.1 Human umbilical vein endothelial cell (HUVEC) lines

Mycoplasma free commercially supplied frozen HUVECs (Cambrex, UK) were reconstituted according to the suppliers instructions into Endothelial Basal Medium (EBM) (Clonetics, UK) supplemented with growth factors including (hydrocortisone, hFGF-B, VEGF, R3-IGF-1, Ascorbic acid, Heparin, hEGF, FBS, GA-1000) (Clonetics, UK) (Figure 2.1b). When cells were confluent, half of the medium was replaced every 2-3 days. For passage, cells were detached using EDTA (0.01%)/Trypsin (0.025%) (Clonetics, UK). Enzyme activity was neutralised using Trypsin neutralising solution (Clonetics, UK). Cells used for subsequent flow cytometry analysis were gently detached with EDTA/PBS (0.1%) without trypsin to avoid possible loss of some cell surface marker expression.

2.1.3.2 Hybridoma endothelial cell line EA-hy-926

Mycoplasma free EA-hy-926 endothelial cells were a gift from Dr CJS Edgell. Frozen cells were reconstituted in Iscove's Modified Dulbecco Media (IMDM) supplemented with 10% fetal calf serum (FCS) (Biowhittaker,UK), 1% Antibiotic (penicillin/streptomycin) (10000Units/ml /50ug/ml) respectively (Sigma, UK), 1x Non-Essential Amino Acids (NEAA) (Sigma, UK), $5x10^{-4}$ M 2-Mercapto-ethanol (2-Me) (Sigma, UK), 1mM sodium pyruvate (Sigma, UK), and 2mM glutamine (Sigma, UK),

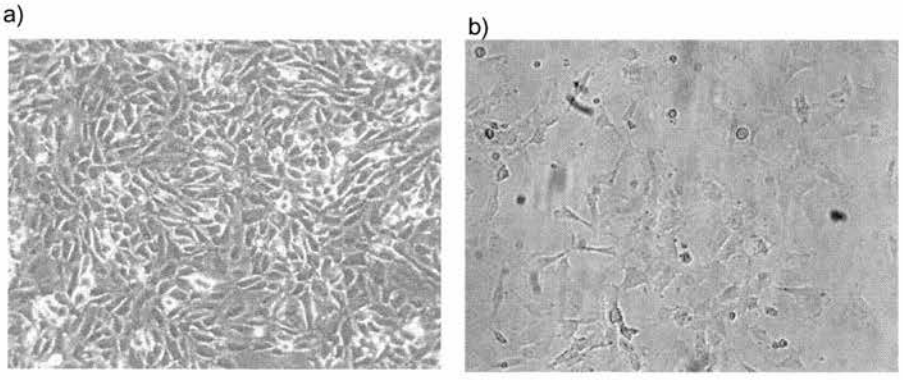


Figure 2.1 Endothelial cell lines

a) Ea.Hy 926 cells cultured in IMDM and b) HUVEC cultured in EBM

(Figure 2.1a). Medium was changed every 2-3 days or when cells were confluent, adding back half of the old medium to half of the new medium. The cells were detached by EDTA/Trypsin (TVP) *(see reagent list)*. Cells used for subsequent flow cytometry analysis were detached by EDTA/PBS (0.1%) without trypsin to avoid possible loss of some cell surface marker expression.

2.1.4 Preservation of cells by freezing

0.5ml freezing medium (10% DMSO and 90% FCS) was gently added to 0.5 ml mononuclear cells ($1x10^6$ to $5x10^7$ cells/ml) suspended in complete culture medium in 1.8ml DNAse and RNAse free tubes (Greiner, UK). Samples were frozen slowly by placing at –20C for 1 hour, transferred to –80C overnight and stored at –140C in a freezer.

2.2 Cell subpopulation isolation

2.2.1 Mononuclear cell isolation

Mononuclear cells (MNC) were separated by buoyant density centrifugation of blood or marrow samples over Histopaque (1.077g/ml; Sigma Diagnostics, UK). Samples were normally diluted 1:2 in PBS, layered over an equal volume of Histopaque (in 15mL or 50mL polypropylene centrifuge tubes) and spun at 400g for 20 minutes. Interface leucocytes were collected and washed twice in phosphate buffer saline (PBS), counted and immediately transferred to IMDM. Unless otherwise stated, isolated mononuclear leucocytes were the basis of subsequent cell subpopulation isolations.

2.2.2 Polymorphonuclear cell isolation

Polymorphonuclear leucocytes (PMNC) were used in bioluminescence studies. Mature peripheral blood neutrophils were isolated from healthy adult venous blood using Polymorphprep (Axis-Shield PC AS, UK). Polymorphprep is a solution for the isolation of polymorphonuclear granulocytes from whole blood. The mononuclear and polymorphonuclear leucocytes are separated into distinct bands free from red cells. The top band at the sample/medium interface consists of mononuclear cells and the lower band consists of polymorphonuclear cells. Carefully, 5ml of freshly-donated blood was layered over 5ml of Polymorphprep solution and

centrifuged at 250g for 30 minutes. The lower polymophonuclear layer was recovered, washed twice in PBS and resuspended in IMDM medium without phenol red.

2.2.3 Purification of CD34$^+$ cells

The CD34-positive subpopulation of mononuclear cells was enriched using either positive selection (MACS CD34 isolation Kit; Miltenyi Biotec, Surrey, UK) or negative selection (Rosette-Sep, Stem Cell Technologies, UK), according to the manufacturers instructions. The efficiency of the purification of CD34$^+$ cells was verified by flow cytometry phenotyping analysis.

2.2.3.1 CD34 progenitor cell enrichment by depletion of mature leucocytes

The Rosette-Sep method (Stem Cell Technologies, UK) is a negative selection cell separation procedure for the isolation of cell subpopulations directly from human whole blood by removal of unwanted cells. It combines the specificity of antibody-mediated cell separation with buoyant density centrifugation. Rosette-Sep is based on complexes of monoclonal antibodies specific for a cell surface marker coupled to monoclonal antibodies specific for red cells (glycophorin-A), to give bispecific tetrameric binding capacity which binds red cells as rosettes around cells expressing the surface marker. For immature HSC isolation, cocktails of tetrameric monoclonal antibodies specific for mature leucocyte markers and red cells are used. The specificities for mature human peripheral blood leucocytes markers include CD2 (T cells, NK cells), CD3 (T cells), CD14 (monocytes), CD16 (neutrophils and NK cells), CD19 (B cells), CD24 (B cells and granulocutes), CD56 (NK cells), CD66b (granulocytes). An adaptation of the manufacturer's Rosette method was used. 2ml of unseparated blood was retained: meanwhile the rest of the blood was diluted and layered over 15 ml of Histopaque (1.077g/ml; Sigma Diagnostics, UK), centrifuged at 500g for 20 minutes and the interface MNC layer was collected and washed in PBS. The retained 2ml of blood and 75ul of the Rosette-Sep antibody cocktail were then added to the mononuclear cells. After 20 minutes incubation at room temperature the unwanted mature cells are cross-linked to red blood cells by the bi-specific antibodies in the Rosette-Sep cocktail. The sample was then centrifuged as above over buoyant density medium (Histopaque), and the unwanted (rosetted) cells pellet al.ong with the free RBCs, leaving the desired cells untouched and highly enriched at the Histopaque-medium interface.

2.2.3.2 CD34 progenitor cell enrichment by selection with immunomagnetic beads

MACS® MicroBeads (Miltenyi Biotech) are superparamagnetic particles that are coupled to specific monoclonal antibodies. They are used to magnetically label the target cell population. The isolation by positive selection of CD34-expressing cells was performed using anti-CD34 antibody coupled to magnetic microbeads. The $CD34^+$ haematopoietic progenitor cells in MNC isolates were magnetically labelled using MACS $CD34^+$ Microbeads (Miltenyi Biotech, Surrey, UK). Washed MNC ($1x10^7$) isolated by buoyant density centrifugation were first incubated with 100µl Fc-receptor blocking reagent (Miltenyi Biotech) for 10 minutes at room temperature to inhibit non-specific or Fc-receptor mediated binding of the CD34-MicroBeads to non-target cells. The cells were then labelled by adding 100µl of CD34-Microbeads, mixed well, and incubated for 30 minutes at 4C. The cells were washed and resuspended in the appropriate volume of medium ready for magnetic separation. The magnetically labelled cells were retained and thus enriched on magnetic columns: meanwhile the cells that did not bind to the CD34 antibody passed through the column. Removal of the column from the magnetic field allowed retained cells to be eluted. To improve purity the recovered $CD34^+$ cells were passed through a second magnetic isolation column.

2.2.4 General immunomagnetic bead enrichment procedure

Specific sub-populations of MNC were isolated by magnetic activated cell sorting (MACS) either directly using specific MAb (CD34, CD133) as described above for CD34 or indirectly using anti-fluorochrome (phycoerythrin) antibody (anti-PE) conjugated to immunomagnetic beads (Miltenyi Biotech, Surrey, UK). For indirect labelling the process was essentially the same. The cells were first labelled with specific-fluorochrome labelled MAb of interest, washed and then labelled with appropriate microbead-conjugated anti-fluorochrome MAbs. Labelled cells were washed before loading to the column. Finally purity of the recovered population was assessed by flow cytometry.

2.2.5 Isolation of short-term (2h) plastic-adherent mononuclear cells

Mononuclear cells isolated by Histopaque buoyant density centrifugation were washed twice with PBS and resuspended in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% FCS (Biowhittaker, UK) and 1% Pen/Strep (Sigma, UK). Approximately $30x10^6$ MNC were plated in a $25cm^2$ tissue culture flask (430168

polystyrene; Corning, UK) and incubated for 2h at 37C. After 2 hours the non-adherent cells were aspirated and set aside. 5ml of PBS were added and aspirated: this procedure was repeated twice. The aspirated cells were added to the non-adherent cell fraction and characterised. Adherent cells were detached using 1ml EDTA/ trypsin for 10 minutes at 37C. 5ml of IMDM with 10% FCS was added to neutralise EDTA/trypsin activity. After washing harvested cells were resuspended in IMDM and characterised by flow cytometry and used for further experiments.

2.2.6 Isolation of cells by fluorescence activated cell sorting (FACS)

100ul of pelleted MNC or 2h plastic adherent cells were directly stained using 5μl of the appropriate anti-human monoclonal antibodies (MAbs) conjugated to phycoerythrin (PE), fluorescein isothiocynate (FITC) or Peridin Chlorophylla protein (PerCP) (see flow cytometry methods, 2.3.1) for 30 minutes in the dark. Samples were then centrifuged at 200g for 10 minutes and washed twice with PBS. Appropriate antibody combination controls were used to set selection gates for FACS sorting. Cells were sorted using a FACS Aria flow cytometer (Becton Dickinson, UK) equipped with 488nm and 633nm lasers. Dead cells were excluded using an electronic SSC/FSC gate before applying sort gates to define cell populations to be collected. A small sample of each collected fraction was re-run through the cytometer to assess the success of sorting by measuring purity of the sample. Sorted populations were recovered and characterised by further analysis.

2.3 Cell identification and phenotyping

2.3.1 Flow cytometry analysis

In general, distributions of cells were investigated in original unmanipulated samples such as whole blood before these were disturbed by cell isolation and enrichment procedures. Various MNC isolates and subpopulations such as $CD34^+$-enriched cells or cells recovered from culture were also phenotyped by flow cytometry. Three colour analysis using a 488nm laser for excitation employed phycoerythrin (PE), fluorescein isothiocynate (FITC) or peridin chlorophylla protein (PerCP) fluorochrome antibody conjugates: four colour analysis was accomplished by adding 633nm excitation to the above and employing allophycocyanin (APC) fluorochrome antibody conjugates.

MAbs used for analysis included anti-CD34-PE and anti-CD34-FITC (Becton

Dickinson, Oxford, UK); anti-CD41-FITC (Becton Dickinson); anti-CD16b-FITC (Immunotech, UK); anti-CD45-PercP (Becton Dickinson); anti-VEGFR2-PE (R&D systems); anti-Ve-cadherin-PE (Santa Cruz Biotechnology); and anti-CD133-APC (Miltenyi Biotec, UK) *(see complete antibody list)*.

2.3.1.1 Simple CD34⁺ population analysis

Cells were stained with PerCP-conjugated anti-human CD45 and PE-conjugated anti-human CD34, fixed and gated for $CD45^{+}CD34^{+}$ cells with a low side scatter, according to the ISHAGE CD34 enumeration protocol (Sutherland et al., 1996) (Figure 2.2).

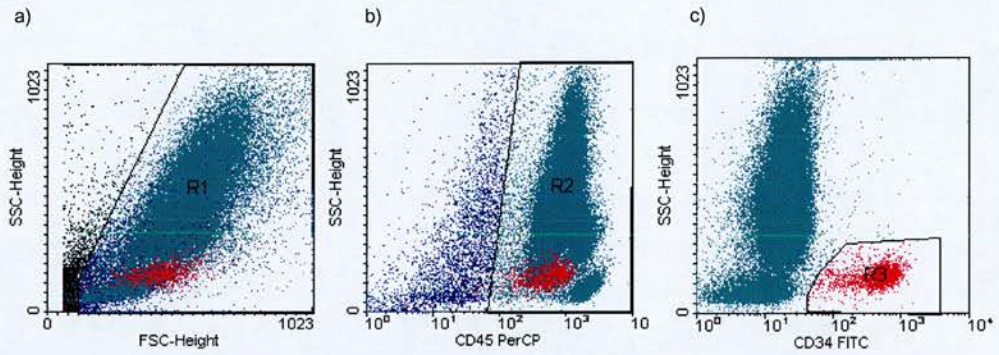


Figure 2.2 CD34 enumeration protocol.

Cells were stained with PercP-conjugated anti-human CD45 and FITC-conjugated anti-human CD34, fixed and gated for $CD45^{+}$ cells co-expressing CD34 with a low side scatter, according to the ISHAGE CD34 enumeration protocol (Sutherland *et al*, 1996). a) Primary gate (R1) set on forward/ side scatter properties (cell size and granularity), b) Expression of CD45 haematopoietic marker (R2). CD45-negative cells here are mainly unlysed red cells, c) CD34-postive gate (R3)(magenta) set in the CD34-positive area. In a) and b) also in magenta $CD34^{+}$ cells are backgated in the SSC/ FSC and in the CD45 axis

In early studies various isotype-matched negative control (non-binding) antibodies were compared with unstained samples, and since these did not differ, unstained samples were used to establish positive stain boundaries. The protocol for staining comprised the following tubes (1) no antibody; (2) anti-CD45 alone; (3) anti-CD45 with anti-CD34. 100ul of sample (usually whole blood) was placed in a Falcon-type 5 ml tube and stained with each appropriate antibody (normally 5ul of undiluted antibody) for 30 minutes in the dark. Where present, as in whole blood analysis, erythrocytes were lysed by FacsLyse (Becton Dickinson, UK) (1 ml for 15 minutes), and cells were spun down and washed at least twice with PBS at 200g (until free of traces of haemoglobin in supernatant or pellet) with blotting of tube mouths to fully remove decanted wash supernatants. The cells were resuspended in 0.5 ml of

CellFix (Becton Dickinson, UK) and analysed within 24h (analysed immediately or stored in the dark at 4C). Events were collected by a FACS Calibur flow cytometer linked to an Apple Macintosh computer equipped with CellQuest software (Becton Dickinson) and calibrated and compensated using Becton Dickinson calibration beads and software for the fluorochromes employed. Samples within any series employed identical stored collection settings. In general collection regions were set on forward-scatter v side scatter for all leucocytes, and on CD45 v side scatter for all leucocytes and a collection gate was set on a combination of these two regions. At least (normally) 50,000 events were counted through this gate, but all events (above the debris exclusion barrier) were saved to allow inspection of CD45-negative events. Data was saved to listmode files and moved to PC (Windows) computers for detailed analysis, using FCS Express software (De Novo Software, www.denovosoftware.com). In some cases data was represented graphically using the WinMDI 2.8 free software for the PC (Windows) developed by Joe Trotter (http://facs.scripps.edu/software.html).

2.3.1.2 Complex immunophenotyping

Essentially the same procedure as for simple $CD34^+$ population analysis (above, 2.3.1.1) was carried out, but with a greater range of antibodies and tubes in the protocol. The MAbs used included anti-CD34-FITC; anti-CD34-APC, anti-CD45-PerCP, anti-CD45-FITC, anti-VEGFR2-PE; anti-CD3-PE, anti-CD19-PE, anti-CD16-PE, anti-CD8-FITC, anti-CD14-PE, anti-CD14-FITC, anti-VE-cadherin-PE; and anti-CD133-APC, anti-CD146-FITC, anti-CD29-FITC, anti-CXCR4-PercP, anti-CD31-FITC, anti-CD63-PE, anti-CD105-APC and anti-Glycophorane-1 (Gpa-1)-PE (see complete antibody list).

2.3.1.3 Staining for intracytoplasmic myeloperoxidase (MPO)

Anti-myeloperoxidase, MPO-7 MAb (Dako Cytomation Ltd, Cambridgeshire, UK), strongly labels the cytoplasm of mature and immature neutrophils. Monocytes are weakly positive while eosinophils are unreactive. Samples (50ul) were stained with anti-CD34-PE and anti-CD45-PercP as above, then fixed using 100ul Dako Intrastain Reagent A (fixation), incubated and washed with PBS. 100ul Dako Intrastain Reagent B (permeabilisation) and 5-10ul of the anti-MPO-FITC cytoplasmic antibody were then added, mixed, washed and resuspended in an appropriate buffer (0.5 ml) for flow cytometry analysis.

2.3.1.4 DiI-Ac-LDL uptake

Acetylated Low Density Lipoprotein (DiI-Ac-LDL), labelled with 1,1'-dioctadecyl – 3,3,3',3'-tetramethyl-indocarbocyanine perchlorate, labels both vascular endothelial cells and macrophages. DiI-Ac-LDL staining kit (Biomedical technologies; UK) was diluted in medium to a concentration of 10ug/ml. Adherent cells were incubated with DiI-Ac-LDL for 4h at 37C in IMDM without FCS and washed several times with PBS. The staining was observed using a fluorescence microscope with FITC filters. Adherent cells were washed and detached with EDTA/trypsin for 10 minutes at 37C. Complete medium was added to neutralise EDTA/trypsin activity and the harvested cells were washed and analysed by flow cytometry. Positive staining was assessed by comparison with unstained cells.

2.3.1.5 *Ulex europaeus* agglutinin-1(UEA-1) lectin binding

Cells were stained with 500ul of 10ug/ul of FITC conjugated UEA-1 (*Ulex europaeus* agglutinin-1) (Sigma, UK) for 1h at 37C. Cells were analysed by flow cytometry. Positive staining was assessed by comparing with an unstained negative control.

2.3.2 Immunofluorescence microscopy

Cells were stained with various endothelial antibodies including anti-VEGFR2-PE (R&D systems), anti-VE-cadherin-PE (Santa Cruz Biotechnology), anti-CD31-FITC (Becton Dickinson, Oxford, UK), Dil-Ac-LDL-FITC (Biomedical technologies; UK), and UEA-1-FITC (*ulex europaeus* agglutinin-1) (Sigma, UK) were fixed and used to prepare cytospins at a concentration of $5x10^4$ cells/ml. The slides were air dried and fixed with Methanol (Fisher, UK) for at least 15 minutes. Then the cells were visualised by a fluorescence microscope (Zeiss).

2.3.3 Morphological analysis (Giemsa staining)

The morphology of the cells was determined using a standard Wright-Giemsa-stained cytospin preparation (Thermo Shandon,UK). Figure 2.3 shows how the cells were scored according to their degree of neutrophil differentiation

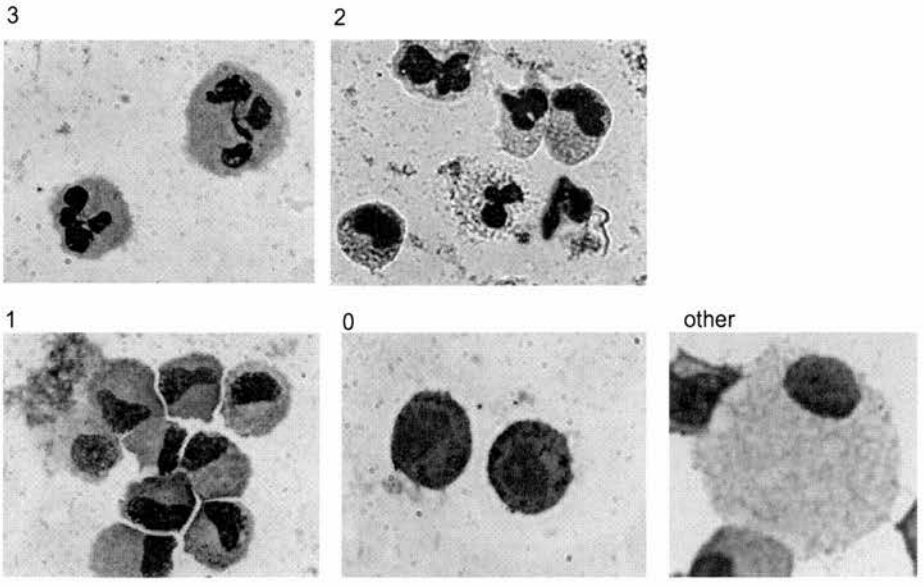


Figure 2.3 Neutrophil morphology scoring.

Pictures show our classification in neutrophil differentiation ranging from 0 (no differentiation) to 3 (most mature). Others represent cells which had no neutrophil or stem cell morphology.

2.4 Bioassays

2.4.1 Colony assays

2.4.1.1 Haematopoietic cell colony assays

Colony-forming cells (CFC) were monitored by their growth in methylcellulose (MC) medium. The use of the Methocult semi-solid matrix with a cocktail of cytokines rhSCF, rhGM-CSF, rhG-CSF, rhIL-3, rhIL-6 and erythropoietin (EPO) allows the proliferation of single haematopoietic progenitors to form distinct colonies containing morphologically recognizable progeny. 50ul of nucleated cells (MNC) were added at $1.5x10^4$ cells, or enriched $CD34^+$ cells at $0.2x10^4$ cells, to 500ul of Methocult containing cytokines (MethoCult GF^+ H4435; Stem Cell Technologies, UK), mixed by aspiration, and plated in 24-well plates (Greiner UK) Colonies (groups of >100 cells) were scored after 14 days according to appearance and counted. The colony-forming unit granulocyte macrophage (CFU-GM), burst-forming unit erythroid (BFU-E), colony-forming unit macrophage (CFU-M) and colony-forming unit granulocyte erythroid, macrophage, megakaryocyte (CFU-GEMM) were

counted after 14 days of culture. Where cells were expanded *ex vivo*, colonies from unmanipulated cells were compared with colonies produced by cells expanded in the presence of various cytokine combinations. Pre-expansion (day 0) CFC were measured on enriched $CD34^+$ cells. Post-expansion (day 14) CFC were measured using recovered expanded cells derived from the cultured $CD34^+$ enriched population, which by this time were generally all CD34-positive following culture in the cytokine combinations studied (any CD34-negative cells did not appear to survive). The results are expressed for each colony type as a percent of total CFC which because of the different starting populations are intended for qualitative interpretation, but for approximate quantitative guidance the colony numbers per thousand cells, and $CD34^+$ cell proportions are also given.

2.4.1.2 Colony Forming Units- Endothelial Progenitor Cells (CFU-EPC).

This assay is based on that described by Hill *et al.*, (2003) but is carried out using commercial kit reagents according to the recommendations of the kit suppliers (Stem Cell Technologies). Mononuclear cells were isolated by buoyant density centrifugation, washed twice with PBS and finally resuspended at $2.5x10^6$ cells/ml in Complete Endothelial Culture Medium (CECM) comprising Endocult Basal Medium (Stem Cell Technologies, UK) supplemented with 1/5 dilution of Endocult supplements (Stem Cell Technologies, UK). Cells were plated at 2ml/well in fibronectin-coated 6-well plates (Becton Dickinson, UK) and incubated for two days at 37C, 5% CO_2 with 95% humidity. After two days when mature endothelial cells and monocytes had adhered (Hill *et al.*, 2003) the non-adherent cells containing the EPC, were counted and pelleted at $0.5\text{-}1x10^6$ cells/ml. 1 ml of fresh CECM was added over the cells and transferred to a fibronectin-coated 24-well plate (Becton Dickinson, UK) for a further three days at 37C, 5% CO_2 with 95% humidity. The colonies per well were then counted and the number of colonies were divided by the number of cells plated in the fibronectin 24-well plate The colonies were defined following the published method (Hill *et al.*, 2003) and Stem Cell Technologies technical manual as a central core of "round" cells with elongated "sprouting" cells at the periphery and are classified as colony forming unit endothelial progenitor cell or CFU-EPC, recently reclassified as early-outgrowth CFU-EPC.

2.4.2 Neutrophil Chemiluminescence assay

Bioluminescence assays were carried out to measure the superoxide respiratory burst activity of freshly isolated peripheral blood neutrophils and of $CD34^+$ derived cells expanded *ex vivo* with different cytokines. This was measured by detecting luminol-

amplified chemiluminescence responses to phorbol myristate acetate (PMA) (Sigma, UK). 100ul of mature peripheral blood neutrophils or expanded cultured progenitors at $0.5x10^6$ cells/ml in IMDM without phenol red and without FCS were plated into 96-well opaque microplates (Greiner, UK), with 100ul of luminol (1mM) (Sigma, UK) and 100ul of PMA (1ug/ml final concentration) in IMDM, and the emitted light activity (relative light units, rlu) was measured on a microplate luminometer (Labsystems Luminoskan) at 37C at 3 minute intervals over 90 minutes controlled by a computer linked to the luminometer and on which measurements were collected for subsequent processing.

2.4.3 Matrigel tube formation

Matrigel® Matrix (Becton Dickinson) is an endothelial cell matrix (ECM) preparation composed primarily of laminin, collagen IV and a number of growth factors. Matrigel solution is thawed slowly overnight on ice/water at 4C and pipettes and plates are pre-cooled. 300ul of matrigel was added over the pelleted cells (usually endothelial cell lines) ($3\text{-}5 \times 10^4$ cells/well) in a pre-cooled 24-well plate (Greiner, UK) and cultured for 5h-22h at 37C with 5% CO_2. Capillary-like structures and endothelial cell networks were examined by phase-contrast microscopy.

2.5 Cell cultures

2.5.1 *Ex vivo* expansion cultures for neutrophil precursor studies

Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen, Paisley, UK) supplemented with 10% FCS (Sigma, UK) and 1% antibiotic (Pen/Strep, Invitrogen, UK) was used for cell culture. $CD34^+$-enriched MNC ($2x10^5$ total cells per ml) of known $CD34^+$ purity were plated in 24-wells plates in 1ml medium with or without various cytokines. The following recombinant purified human cytokines were used in these studies: flt-3 ligand (Flt3-L), stem cell factor (SCF), thrombopoietin (TPO), interleukin-6 (IL-6) and interleukin-3 (IL-3), all from (PeproTech Ec Ltd, London, UK). Recombinant human granulocyte colony-stimulating factor (G-CSF; lenograstim) was a gift from Chugai Pharma, UK (*See combinations of cytokines for neutrophil precursor studies list*). Cells were cultured in 100ng/ml SCF, 10ng/ml Flt3-L unless otherwise stated, with or without 100 ng/ml G-CSF. Cells were cultured in a fully humidified atmosphere of 5% CO_2 in air at 37ºC, for 14 days. Following a vigorous pipetting, aliquots of non-adherent cells were removed for cell counts,

differential morphology, flow cytometer analysis and colony assays. Few residual adherent cells remained in wells, and those appeared to have stromal morphology. $CD34^+$ numbers were calculated from total cell numbers and $CD34^+$ proportions: $CD34^+$ expansion was expressed as a "fold expansion" over starting $CD34^+$ numbers, where fold expansion is the final number of cells after expansion divided by the initial $CD34^+$ numbers before culture. In most cases cytokine expanded cells exhibited some degree of expression of CD34: cells cultured without cytokines died. It was therefore supposed that the expanded cells derived from the starting $CD34^+$ population and that the starting CD34-negative (mature) MNC did not contribute to the expansion.

2.5.2 *Ex vivo* expansion cultures for endothelial precursor studies

The $CD34^+$ enriched fraction of MNC cell suspensions isolated by magnetic-activated cell sorting (MiniMacs $CD34^+$ isolation Kit; Miltenyi Biotec, Surrey, UK) from peripheral blood, cord blood, mobilised blood and bone marrow were cultured in expansion culture medium (Iscove's Dulbecco's medium; Invitrogen, Paisley, UK) supplemented with 10% FCS (Sigma, UK), and 1% Pen/Strep (Sigma, UK). $0.2x10^6$ total cells/ml of known $CD34^+$ purity were plated in 24-well plates in 1ml medium with the addition of VEGF alone, or with 100ng/ml SCF plus 10ng/ml Flt-3L alone, or with different combinations of cytokines as stated [such as 50-100ng/ml of VEGF, 10ng/ml of bFGF, 100ng/ml of GM-CSF, 10 units of heparin/ml, and 100ng/ml of Endothelial cell growth supplement (ECGS)] *(See combinations of cytokines for endothelial precursor studies list)* in a fully humidified atmosphere of $5\%CO_2$ in air, at 37C for 14 days. Cultured cells were harvested by adding 0.5ml of EDTA/PBS (0.1%) for 15 minutes at room temperature followed by vigorous pipetting to recover adherent and non-adherent cells, which were counted, and characterized by flow cytometry, to assess any expansion. In some cases cells were then plated on fibronectin-coated 24-well plates in CECM medium (Stem cell Technologies, UK) and assessed for the generation of CFU-EPC colonies, and also assessed for endothelial tube formation in Matrigel.

The following recombinant purified human cytokines were used in these studies. Human flt-3 ligand (Flt3-L), human stem cell factor (SCF), human basic Fibroblast growth factor-basic (bFGF), and human Vascular endothelial growth factor (VEGF) were all from (PeproTech Ec Ltd, London, UK). Endothelial cell growth supplement (ECGS) and Heparin were from Sigma, UK. Human granulocyte-monocyte colony stimulating factor (GM-CSF) was from Sandoz, UK. Interleukin-4 was from RD

Systems, UK. Human granulocyte colony-stimulating factor (G-CSF; lenograstim) was a gift from Chugai Pharma, UK. *(See combinations of cytokines for endothelial precursor studies list).*

In some cases fibronectin and gelatin coated dishes were used. In most cases we used a commercial fibronectin coated plated from (Becton Dickinson, UK) but some cases we used our own coating. Here fibronectin (0.5mg/ml) (Sigma, UK) was sterilised by filtration using a 0.22um filter (Millipore, UK), diluted into carbonate/ bicarbonate pH 9.6 buffer at a final concentration of 10ng/ml per well in 24-well plates and incubated overnight at 4C. On the day of the experiment the wells were washed twice with PBS and once with medium with FCS and the cells were plated. Alternatively, for gelatine-coated plates, 500µl of Gelatine (0.1%) (Sigma, UK) was added to the 24 well plates and left for 30 minutes at 37C. The wells were then washed with PBS and the cells were plated. A Clonetics endothelial medium (EBM) was also studied in comparison to IMDM.

2.5.3 Dendritic cell differentiation

MNCs from peripheral blood were incubated in a $25cm^2$ flask (430168 polystyrene) (Corning, UK) with IMDM for 2h as described in 2.2.5. Recovered adherent cells (>80% $CD14^+$) were cultured in a 24 well plate in IMDM in the presence of 100ng/ ml of GM-CSF (Sandoz, UK) and 15ng/ml of IL-4 (RD Systems, UK). After 7 days culture the cells were characterised by flow cytometry immunophenotyping for markers of dendritic cells (CD209, CD58 (Becton Dickinson, UK), CD11c, CD86, CD80, HLA Class I and HLA Class II (Caltag, UK)) and transferred to a 24-fibronectin-coated plate in CECM medium (Stem Cell Technologies) for 5 days to test for the generation of CFU-EPC colonies.

2.6 Other manipulations and analyses of cells

2.6.1 Cell preparation for Western blotting for detection of SHP-1, SHP-2 and PTEN expression

Cells were cultured as for neutrophil precursor studies (above, 2.5.1) with cytokine combinations as stated in results. After appropriate culture times cells were recovered, washed twice with $Ca2^+/Mg2^+$ free PBS. After counting cells were resuspended at $1x10^6$ in 50ul lysis buffer (1% Triton-X 100, 150mM NaCl, 25mM Tris-HCl pH 7.4, 1mM EDTA, plus protease inhibitor cocktail tablets, 1mM NaF, 1mM Na3V04, 1mM Levamisole, 1mM ß-glycerophosphate), mixed and incubated

on ice for 20 minutes. Following centrifugation at 20,000g for 15 minutes the supernatant was transferred to a fresh eppendorf tube, 25ul of double-strength Laemmli buffer (125mM Tris HCl pH 6.8, 4% SDS, 10% beta-mercaptoethanol, 20% Glycerol, and a trace of bromphenol-blue powder) was added and the samples heated for 5 minutes at 95C before storage at –20C.

2.6.2 Western blotting

Protein separation was carried out using 8% SDS-page gels (30% Acrylamide), and transferring the proteins to PVDF membranes. The membranes were washed in PBS, blocked for 1h in 2% casein and incubated with the indicated primary antibody (Transduction Lab) in blocking buffer overnight at 4C. The membrane was then washed for 30 minutes with PBS/Tween (0.05%) and incubated with the anti-mouse HRP secondary antibody for 1h. Finally, after washing for 45 minutes with PBS/ Tween (0.05%) the membrane blot was developed with ECL Plus and digitalised using a Versadoc imaging System (Biorad).

2.6.3 Transfection of *ex vivo* differentiated cells derived from $CD34^+$ cells

Cells were transfected using Amaxa® transfection reagent (Amaxa, Cologne, Germany) according to the manufacturer's protocol. In brief, after appropriate culture times cells were washed with $Ca2^+/Mg2^+$ free PBS and the supernatant was discarded. 100ul of Amaxa buffer was added to the pellet plus 2ug of either SHP-1-GFP-control-GFP or SHP-1-GFP construct. The cDNA encoding human tyrosine phosphatase SHP-1, and SHP-2 were a kind gift from Paul Crocker (University of Dundee, UK) and were cloned into the pEGFP-N1 vector (Clontech, Basingstoke, UK)(Avril *et al.*,2004).The mixture was transferred into a cuvette, and cells were electroporated (Amaxa, Cologne, Germany) using the appropriate program. Using a thin Pasteur pipette cells were recovered, washed and plated in a 12-well plate in pre-warmed RPMI medium overnight. After 20h, apoptosis of the transferred cells was analysed by assessment of phosphatidylserine expression using Annexin-V-PE staining (Caltag, UK) by FACS analysis (Coulter Epics XS). $1x10^5$ cells were washed with PBS and resuspended in 100ul of Annexin buffer (10mM Hepes/NaOH, pH 7.4, 140mM NaCl, 2.5mM $CaCl_2$) plus Annexin-V-PE (Caltag, UK) and incubated for 15 minutes at room temperature in the dark. Cells were washed by centrifugation following addition of 400ul of Annexin buffer and the pellet resuspended in Annexin buffer for analysis.

2.6.4 Plasma analysis

Plasma troponin I concentrations were measured using an automated immunometric assay (Ortho-clinical Diagnostics, High Wycombe, UK). Serum was prepared for measurement of C-reactive protein (CRP) concentrations using an immunonephelometric assay (Behring BN II nephelometer, Marburg, Germany).

2.6.5 RNA extraction and quantitative real-time PCR

Total leucocyte RNA extraction from 1 mL of whole blood was performed using QIAGEN's RNeasy Mini Kit (QIAGEN Ltd., Crawley, UK). RNA concentration and purity were estimated by UV absorbance at 260 and 280 nm. The 260:280 nm ratios were all greater than 1.8 indicating that little protein contamination was present. One microgram of total RNA was transcribed into cDNA in each reverse transcription reaction with 200 units of M-MLV reverse transcriptase for 60 minutes at 37°C in 20 mL reactions containing 1 mL (0.5mg/ml) of random hexamer primers, with 0.625 mL (40 units/mL) of RNAse inhibitor, 5 mL of dNTP mix (containing dATP, dCTP, dGTP and dTTP, each at a concentration of 10 nM in water) and 5 mL of 5X RT reaction buffer (containing 50 mM Tris-HCl pH 8.3 at 25°C, 75 mM KCL, 3 mM $MgCl_2$ and 10 mM DTT).

Real-time PCR was carried out using the ABI Prism â7900HT system (Applied Biosystems, Warrington, UK) to determine the relative quantity of mRNA for selected genes. Relative quantity can be defined as a comparison of a target signal in different samples to a reference sample and normalised to an endogenous control. PCR primers and probes for amplification of cDNA derived from CD34 and CD14, and the mature endothelial markers, VE-cadherin and von Willebrand factor (vWF), transcripts were obtained from Applied Biosystems (Foster City, CA, USA). Each assay contained forward and reverse PCR primers (final concentration of 900 nM each) and one Taqman MGB probe (6-FAM dye-labelled, to a final concentration of 250 nM). All primer-probe sets had been quality control checked and validated. The ribosomal 18s gene was used as an internal control. Four mL of the reverse transcription reaction was analysed in each PCR reaction. The PCR reactions were run in triplicate in 20 mL assays, each containing cDNA, 1 mL of primer/probe, 10 mL of universal PCR mastermix and distilled water. The cycling program was as follows: (a) initial activation for 10 minutes at 95C; (b) 50 amplification cycles with a 15 second denaturing step at 95C, and a 1 minute combined annealing and extension step at 60C. Analysis was performed using ABI 7900HT SDS software (version 2.1) in order to obtain the relative quantities of mRNA compared to a calibrator.

2.7 Statistical analyses

The medians of the different sets of results were compared using non-parametric tests (Wilcoxon matched pairs test or Mann-Whitney test depending on whether pairing was possible). A probability value was considered significant if it was $p<0.05$ (represented in results as $p<0.05$ a'' *, $p<0.01$ a'' ** and $p<0.001$ a'' ***). GraphPad or NCSS statistical packages were used.

2.8 Clinical studies

2.8.1 Coronary angiography and PCI

All patients were treated for two weeks with 75 mg clopidogrel prior to angiography or PCI. Coronary angiography was performed via right femoral or radial artery approach with 6F arterial catheters. Elective PCI was performed in all patients after 7,500 IU intravenous heparin administration and in one patient after intravenous glycoprotein IIb/IIIa inhibitor. Coronary stents (Liberté, Boston Scientific) were implanted in all patients after balloon pre-dilatation of the lesion without apparent procedural complications.

Reagents List

-Acetylated Low Density Lipoprotein (DiI-Ac-LDL) (Biomedical Technologies, UK)
-CellFix (Becton Dickinson, UK)
-Chloroquin (2Mm) (Sigma, UK)
-Chloroquin/EDTA (2Mm/0.1%) (Sigma, UK/ Fisher, UK)
-Dako Intrastain Reagent A (fixation) (Dako Cytomation Ltd)
-Dako Intrastain Reagent B (permeabilisation) (Dako Cytomation Ltd)
-EDTA (0.01%)/Trypsin (0.025%) (Clonetics, UK) (Used only in HUVECs)
-EDTA/PBS (0.1%) (Fisher, UK/ Oxoid, UK)
-FacsLyse (Becton Dickinson, UK)
-Fetal Calf Serum (FCS) (10%) Biowhittaker, UK
-Gelatin (0.1%) Sigma, UK
-Histopaque (1.077g/ml; Sigma Diagnostics, UK).
-Laemmli buffer (125mM Tris HCl pH 6.8, 4% SDS, 10% beta-mercaptoethanol, 20% Glycerol, and a bit of bromphenol-blue powder).
-Lignocain (2Mm) (Lidocain) (Hameln Pharmaceuticals Ltd)
-Lignocain/EDTA (2Mm/0.1%)) (Hameln Pharmaceuticals Ltd/ Fisher, UK)
-Luminol (1Mm) (Sigma, UK)
-Lysis buffer (1% Triton-X 100, 150mM NaCl, 25mM Tris-HCl pH 7.4, 1mM EDTA, plus protease inhibitor cocktail tablets, 1mM NaF, 1mM Na3V04, 1mM Levamisole, 1mM ß-glycerophosphate).
-MACS CD34 Isolation Kit (Miltenyi Biotec, Surrey, UK)
-Matrigel®Matrix (Becton Dickinson, UK)
-Methanol (Fisher, UK)
-PBS/Tween (0.05%) (Oxoid, UK/ Fisher, UK)
-Penicillin/Streptomycin (10.000Units/ml (Penicillin)+ 10ug/ml (Streptomycin) (Sigma, UK).
-Phorbol myristate acetate (PMA) (1ug/ml) (Sigma, UK)
-Phosphate buffered saline (PBS) tablets (Oxoid, UK)
-Polymorphprep (Axis-Shield PC AS, UK)
-Rosette-Sep (Stem Cell Technologies, UK)
-Trypsin neutralising solution (Clonetics, UK). (Used only in HUVECs)
-Trypsin/EDTA (TVP): 0.078g EDTA (Fisher, UK)+ 2.5ml Trypsin (x10 solution 2.5%)(Sigma, UK)+ 2.5ml chick serum (Gibco, UK)+ 245ml of PBS (Oxoid, UK)
-*Ulex europaeus* agglutinin-1 (UEA-1) lectin binding (Sigma,UK)
-Wright-Giemsa staining (Thermo Shandon, UK)

Culture mediums

-Endothelial basal medium (EBM) culture medium (Clonetics, UK) plus added growth supplements (hydrocortisone, hFGF-B, VEGF, R3-IGF-1, Ascorbic acid, Heparin, hEGF, FBS, GA-1000) (Clonetics, UK).

-Freezing medium (10% Dimethyl sulphoxide (DMSO) (Sigma, UK) and 90% FCS)

-Iscove's Modified Dulbecco Media (IMDM) with 10% FCS (Biowhittaker,UK) 1% Antibiotic (penicillin/streptomycin) (10000Units/ml /10ug/ml)respectively (Sigma, UK).

-Iscove's Modified Dulbecco Media (IMDM) with 10% FCS (Biowhittaker,UK) 1% Antibiotic (penicillin/streptomycin) (10000Units/ml /10ug/ml)respectively (Sigma, UK), 1x Non-essential aminoacids (NEAA) (Sigma, UK), $5x10^{-4}$ M of 2-Mercapto-ethanol (2-ME) (Sigma, UK), 1mM sodium pyruvate (Sigma, UK), and 2mM glutamine (Sigma, UK), (Hybridoma endothelial cell line EA-hy-926 only).

-Mesencult media (Stem Cell Technologies, UK) supplemented with 10% FCS (Biowhittaker,UK), 2mM glutamine (Sigma, UK), and 1% Antibiotic (penicillin/ streptomycin) (10000Units/ml /10ug/ml)respectively (Sigma, UK).

Colony assay mediums

-Complete Endothelial Culture Medium (CECM) comprising Endocult Basal Medium (Stem Cell Technologies, UK) supplemented with 1/5 dilution of Endocult supplements (Stem Cell Technologies, UK).

-MethoCult GF^{+} H4435; Stem Cell Technologies, UK

List of antibodies used

anti-CD105-APC (Caltag, UK)
anti-CD11c-PE (Caltag, UK)
anti-CD133-APC (Miltenyi Biotec,UK)
anti-CD146-FITC (RD Systems, UK)
anti-CD14-FITC (Becton Dickinson,UK)
anti-CD14-PE (Becton Dickinson, UK)
anti-CD16b-FITC (Immunotech)
anti-CD16-PE (Becton Dickinson,UK)
anti-CD19-PE (Becton Dickinson,UK)
anti-CD209-PE (Becton Dickinson, UK)
anti-CD29-FITC (Caltag, UK)
anti-CD31-FITC (Becton Dickinson, UK)

anti-CD34-APC (Becton Dickinson,UK)
anti-CD34-FITC ((Becton Dickinson,UK)
anti-CD34-PE (Becton Dickinson,UK)
anti-CD3-PE (Becton Dickinson, UK)
anti-CD41-FITC (Becton Dickinson, UK)
anti-CD45-FITC (Becton Dickinson, UK)
anti-CD45-PercP (Becton Dickinson, UK)
anti-CD58-FITC (Becton Dickinson, UK)
anti-CD63-PE (Caltag, UK)
anti-CD80-PE (Caltag, UK)
anti-CD86-FITC (Caltag, UK)
anti-CXCR4-PercP (Becton Dickinson, UK)
anti-Glycophorane-1 (Gpa-1)-PE (Serotec, UK)
anti-HLA-Class I-FITC (Caltag, UK)
anti-HLA-Class II-PercP (Caltag, UK)
anti-myeloperoxidase (MPO-7 Mab) (Dako Cytomation Ltd)
anti-Ve-cadherin-PE (Santa Cruz Biotechnology)
anti-VEGFR2-PE (R&D systems)

Combinations of cytokines for neutrophil precursor studies.

SCF (10,100 ng/ml) (PeproTech Ec Ltd, UK)
Flt3-L (10,100 ng/ml) (PeproTech Ec Ltd, UK)
SCF (10,100 ng/ml)+ Flt3-L (10,100 ng/ml)
TPO (10,100 ng/ml) (PeproTech Ec Ltd, UK)
SCF (100 ng/ml)+ Flt3-L (10 ng/ml)+ TPO (10,100 ng/ml)
SCF (100 ng/ml)+ Flt3-L (10 ng/ml)+ IL-6 (10ng/ml) (PeproTech Ec Ltd, UK)
SCF (100 ng/ml)+ Flt3-L (10 ng/ml)+ IL-3 (10ng/ml) (PeproTech Ec Ltd, UK)
SCF (100 ng/ml)+ Flt3-L (10 ng/ml)+ IL-6 (10ng/ml) + IL-3 (10ng/ml)
G-CSF (lenograstim) (10,100 ng/ml) (gift from Chugai Pharma, UK)
SCF (100 ng/ml)+ Flt3-L (10 ng/ml)+ G-CSF (10,100 ng/ml)
SCF (100 ng/ml)+ G-CSF (100 ng/ml) + TPO (100 ng/ml)
SCF (100 ng/ml)+ Flt3-L (10 ng/ml)+ G-CSF (100 ng/ml) + TPO (100 ng/ml)
SCF (100 ng/ml)+ Flt3-L (10 ng/ml)+ G-CSF (100 ng/ml))+ IL-6 (10ng/ml)
SCF (100 ng/ml)+ Flt3-L (10 ng/ml)+ G-CSF (100 ng/ml))+ IL-3 (10ng/ml)
SCF (100 ng/ml)+ Flt3-L (10 ng/ml)+ G-CSF (100 ng/ml))+ IL-6 (10ng/ml)+ IL-3 (10ng/ml).

Combinations of cytokines for endothelial precursor studies.

VEGF (50,100ng/ml) (PeproTech Ec Ltd, UK)

SCF (100 ng/ml)+ Flt3-L (10 ng/ml)+ VEGF (50,100ng/ml)

SCF (100 ng/ml)+ Flt3-L (10 ng/ml)+ VEGF (50,100ng/ml)+10 ng/ml bFGF (PeproTech Ec Ltd, UK)

SCF (100 ng/ml)+ Flt3-L (10 ng/ml)+ VEGF (50,100ng/ml)+10 Units Heparin/ml (Sigma, UK)

SCF (100 ng/ml)+ Flt3-L (10 ng/ml)+ VEGF (50,100ng/ml)+100ng/ml Endothelial growth factor supplement (ECGS) (Sigma, UK)

SCF (100 ng/ml)+ Flt3-L (10 ng/ml)+ GM-CSF (100ng/ml) (Sandoz,UK)

GM-CSF (100ng/ml) (Sandoz, UK) +15 ng/ml IL-4 (RD Systems, UK)

Chapter 3:

Formation of functional neutrophil precursors from human peripheral blood haematopoietic stem cells.

3.1 Research aims

The principal aim of this section of the project was to examine at a laboratory level the feasibility of supporting mobilised peripheral blood stem cell (PBSC) transplantation by setting aside part of the PBSC harvest for *ex vivo* manipulation to achieve accelerated *in vivo* neutrophil recovery. A subordinate aim was also to explore conditions for *ex vivo* manipulation to achieve simultaneous accelerated *in vivo* platelet recovery. These investigations were intended to inform preclinical translation of the findings from research laboratory to good manufacturing practice (GMP) grade laboratory facility for development for possible clinical application.

3.2 Introduction

3.2.1 Therapeutic use of Haematopoietic stem cells

The concept of regenerative medicine using the body's own or histocompatible donor haematopoietic stem cells for a rapid haematological reconstitution has been studied and applied as a treatment of haematological disorders. Myeloablation by chemotherapy followed up by haematopoietic stem/progenitor cell transplantation is indicated in the treatment of a wide variety of leukaemias, lymphoproliferative diseases, solid tumours, bone marrow failure syndromes, immunodeficiency states and non-malignant haematological disorders (Barrett, 1991). Neutrophils are one of the body's first line of defences and work by going to the site of infections, damage, or inflammation causing phagocytosis of particles such as bacteria. Patients receiving

high doses of chemotherapy are at risk for serious infections and bleeding during the neutropenia and thrombocytopaenia phase induced by their treatment.

3.2.2 Autologous and allogeneic haematopoietic stem cell transplantation

Autologous (from the same person) and allogeneic (from a histocompatible donor) haematopoietic stem cell transplantation have been used successfully for more than three decades in the treatment of haematological diseases to reconstitute haematopoiesis after myeloablation (Verfaillie, 2002).

3.2.3 Different sources of HSC for transplantation.

The classic source of haematopoietic stem cells is the bone marrow. For more than 40 years, clinicians performed bone marrow transplants from patients (autologous) or donors (allogeneic) as an effective form to restore haematopoiesis following high doses of chemotherapy (Chao and Blume, 1989; Chao and Blume, 1990). The development of bone marrow transplantation provided a method where higher doses of chemotherapy could occur (not possible with previous therapies limited by bone marrow toxicity) and therefore higher tumour cell kill with expected better outcomes (Chao and Blume, 1989). Alternatively, the use of peripheral stem cells has also been studied. In 1979 Goldman reported successful autologous peripheral blood stem cell transplantation in patients with chronic myelogenous leukaemia (Goldman, 1979). However, circulating human HSC in the bloodstream are very low. In the recent years researchers have found that by injecting the cytokine granulocyte-colony stimulating factor (G-CSF) a few days before the cell harvest, haematopoietic stem cells can be mobilised in significant numbers and migrate from marrow to blood increasing the numbers of stem/progenitor cells in peripheral blood (www.nih.gov/news/stemcell/scireport.htm). Currently, cytokine mobilised peripheral blood stem cells (PBSC) are replacing bone marrow to become the most common source of HSC for transplantation, generally with a reduced period of post transplant neutropenia and thrombocytopenia (To *et al.*, 1992; Hartmann *et al.*, 1997; Paquette *et al.*, 2000). This method reduces complications associated with high doses of chemotherapy, shortens hospital stay, decreases costs, and accelerates and enhances haematopoietic recovery (www.nih.gov/news/stemcell/scireport.htm). Alternatively, in the late 1980s and early 1990s, researchers began to recognise that blood from human umbilical cord was also a rich source of HSCs for allogeneic transplantation. HSCs derived from cord blood (CB) seem to have many advantages for transplantation, but the recipients are restricted to paediatric patients because

of the small cord blood volume (Cairo and Wagner, 1997; Rubinstein *et al.* 1998).

3.2.4 The period of neutropenia following PBSC HSC transplantation.

Myeloablative chemotherapy destroys the bone marrow's capacity for haematopoiesis. Infusion of $CD34^+$ HSC harvested pre-myeloablation following G-CSF mobilisation restores haematopoiesis allowing more aggressive chemotherapy. However HSC transplant does not immediately restore completely mature blood cells. There is a period of neutropenia until the restorative HSC transplant takes effect. The neutropenia following HSC transplant can result in life-treating infections and plays an important role in the design, schedule, and doses of cancer treatment regimes (Tutschka, 1986; Ringden *et al.,* 1988). The development of an optimal therapy to augment this recovery will be a great advantage (Bender; Smith and Unverzagt, 1995). A previous approach to the treatment of neutropenia included granulocyte transfusions (Pizzo, 1984). However, this therapy was not widely used because of the difficulties in obtaining adequate granulocytes from allogeneic donors, their short half-life, impossible storage and the potential of the transmission of infections (Strauss, 1993).

The HSCs identified by the CD34 cell surface marker (Sutherland *et al.*, 1996), are commonly mobilised into peripheral blood by administration of G-CSF (lenograstim) (Thomas *et al.*, 2002). A cell harvest is collected by aphaeresis (on a cell separator) when appropriate numbers of $CD34^+$ cells are present in peripheral blood. Locally, for autologous peripheral blood stem cell transplantation $>2.5x10^6$ $CD34^+$ cells/kg is felt to be an adequate dose. This is similar for allogeneic PBSCT although the ideal dose is $5x10^6$ $CD34^+$ cells/kg with greatest benefit for graft versus host disease (GVHD) (H.R Consultant Haematologist, personal communication). Peripheral blood stem cell harvests are then stored frozen (the $CD34^+$ cells can survive intact) and recovered for administration at the appropriate time, following intense chemotherapy. The stem cell source may be autologous (collected from the patient after initial chemotherapy-induced remission) or allogeneic, from a histocompatible donor (usually a relative) not in receipt of chemotherapy. Cytokine mobilised peripheral blood stem cell transplantation after chemotherapy shortens the period of neutropenia when compared to whole bone marrow (Kasai *et al.*, 2002). However, there remains a period of many days, about 10 to 15 days, of clinically significant neutropenia, during which the patient is at high risk of infection and which cannot be reduced by increasing $CD34^+$ doses (Thomas *et al.*, 2002). Thus, this neutropenia period is probably related to the normal maturation time for the re-infused cells to

proliferate and differentiate into mature cells *in vivo*.

3.2.5 The period of thrombocytopaenia following PBSC HSC transplantation.

Thrombocytopaenia is a disorder of reduced platelet counts in which the patients are at high risk of serious bleeding (Geissler *et al.*, 2003). Support for thrombocytopaenia is routinely available from transfusion centres through allogeneic leukodepleted platelet transfusions, which present low risk of virus transmission since they are not nucleated cells (To *et al.*, 1992). Prolonged and severe chemotherapy-induced thrombocytopenia is a major cause of morbidity in patients receiving intensive chemotherapy and especially in those undergoing stem cell transplantation following intensive myeloablative chemotherapy (Tornebohm *et al.*, 1993). Following HSC transplantation platelet engraftment requires 11-25 days, so extends beyond the period of neutropenia. This has prompted a search for a growth factor that stimulates platelet production, comparable to G-CSF for neutrophils or erythropoietin (EPO) for red cells. Thrombopoietin (TPO) or megakaryocyte growth and development factor (MGDF) is the most specific and effective growth factor identified to date for the treatment of thrompocytopaenia. Exogenously administered MGDF might further stimulate megakaryogenesis and platelet production (Nomura *et al.*, 2002). However, Li *et al.* (2001) showed that a small but significant proportion of subjects resulted in thrombocytopaenia due to the development of neutralising antibodies to endogenous TPO.

3.2.6 The residual period of neutropaenia and thrombocytopaenia. A proposal to reduce it by *ex vivo* haematopoietic stem cell expansion.

Haematopoietic stem cell transplantation after chemotherapy resolves neutropenia and thrombocytopenia but there remains a period of many days of clinically significant lack of functional numbers of neutrophils and platelets. Selective manipulation of the cellular composition of the grafts may offer the potential for reducing the post-transplant period of neutropenia and thrombocytopaenia, thus reducing the morbidity and mortality generally associated with these therapies (Noga, 1992; Berenson *et al.*, 1995). The availability of recombinant cytokines has allowed investigation of the role of different cytokines in driving *ex vivo* proliferation and maturation of $CD34^+$ cells with different haematopoietic potential, and investigation of the use of different combinations of cytokines for expansion of HSC for different clinical objectives. This approach first proposed by Haylock *et al.*,

1992 was to give unmanipulated HSC to ensure the long-term haematopoietic engraftment and as an adjunct to give *ex vivo* expanded HSC for reduction of the period of neutropenia. However, many studies have targeted *ex vivo* expansion of the entire graft, including all classes of HSC encompassing primitive long-term reconstituting cells and mature lineage-committed progenitors, to provide sufficient reconstitutive graft material from smaller starting amounts of PBSC, without support from unmanipulated PBSC. However, Holyoake *et al.*, 1997 demonstrated clinically that transplantation solely with *ex vivo* expanded $CD34^+$ cells does not confer durable haematopoietic reconstitution, and that unmanipulated PBSC are required for durable reconstitution. Of necessity this remains the target of advocates of the use of umbilical cord blood HSC for allogeneic transplantation where due to volume limitations HSC numbers are too low for use in adults.

Some studies have focussed, like Haylock, on using only part of the PBSC harvest to deliberately expand neutrophils. Here there is no critical requirement in culture at least for maintenance, at best expansion, of long-term haematopoietic potential which are in such cases given by the unmanipulated harvest component of the graft. However, most *ex vivo* protocols and preclinical trials, have aimed to achieve simultaneous expansion of both neutrophil and megakaryocytes precursors to address the dual problems of neutropenia and thrombocytopenia (McNiece *et al.*, 2000; Paquette *et al.*, 2000; Prince *et al.*, 2004; Reiffers *et al.*, 1999). Reviews of such studies (Heike and Nakahata, 2002; McNiece and Briddell, 2001; McNiece, 2004) affirm the safety of the infused manipulated material and generally more rapid neutrophil engraftment than historical controls, even resulting in abolition of neutropenia in some cases Reiffers *et al.*, 1999, which appears to support some role for *ex vivo* expansion of neutrophil precursors in further reducing neutropenia. However, with few exceptions, such "dual expansion" *ex vivo* manipulated cells have not demonstrated any significant effect on thrombocytopenia. There are few clinical studies which have assessed the efficacy of selectively expanded megakaryocyte progenitors and none yet which have shown any clinical benefit in reducing thrombocytopaenia as an adjunct to autologous PBSC transplantation (Bertolini *et al.*, 1997; Decaudin *et al.*, 2004, Scheding S *et al.*, 2004).

Further studies and new combinations of cytokines are needed to determine the optimal *ex vivo* protocol application for the treatment of thrombocytopenia.

Platelet transfusions are currently available whereas there are only few centres offering routine granulocyte transfusion. There have been some laboratory studies which have focussed on the selective generation of neutrophil progenitors from cord blood (De Bruyn *et al.*, 2003) or PBSC (Hino *et al.*, 2000; Scheding *et al.*, 2000;

Brugger *et al.*, 1993) but the cytokine combinations used were complex, and these have not been tested clinically.

Also, in the scale-up of any preclinical procedure for clinical use it is necessary to be aware of good manufacturing practice, cost-benefit and regulatory issues (Giordano *et al.*, 2004; Wall and Prince, 2003). To date, part of the reason none of these *ex vivo* protocols has been adopted for routine clinical use may be because most of them do not compare favourably on a cost-benefit basis to conventional support for HSC transplantation such as transfusion of blood or blood components or antibiotic administration for 5 to 7 days. However, support for neutropenia by allogeneic donor granulocyte transfusion is not routinely available and neutropenia is still a major cause of death as patients remain at high risk from life-threatening infections. In this context, it may be important to examine specific expansion of neutrophil precursors from autologous PBSC, as an intended adjunct to unmanipulated autologous PBSC transplantation. We therefore undertook a study to examine selective generation of neutrophil progenitors from PBSC which might rationalise and simplify the combination of cytokines employed, with a view to clinical development as an adjunct to autologous PBSC transplantation for reduction of neutropaenia.

3.3 Results

3.3.1 CD34⁺ cell enrichment

CD34⁺ cells were enriched to a purity of around 15-65% using the Rosette-Sep (negative-selection) method or 60-90% using the Miltenyi (positive selection) method. The negative selection method might retain primitive CD34-negative HSC. Figure 3.1 illustrates side by side the two selection methods, showing the different cell populations and the degree of CD34⁺ stem cell purification. Improved yields of CD34⁺ cells were obtained by the positive-selection method, which was used in most cases, but similar outcomes were obtained with CD34⁺ cells enriched by the negative selection method and these were included in the results.

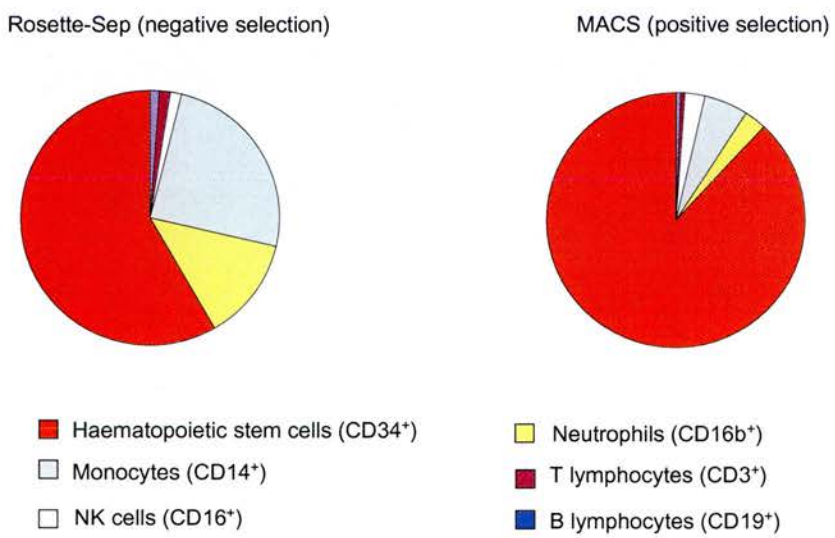


Figure 3.1 CD34⁺ cell enrichment.

Comparison of an individual example between Rosette-Sep (CD34⁺ negative selection) with MACS (CD34⁺ positive selection) both described in the materials and methods (see 2.2.3). Higher purity of CD34⁺ cells was obtained by the positive-selection method. However some residual mature cell populations still remained. Blood source: G-CSF mobilised peripheral blood.

3.3.2 Phenotype of the initial cell population before expansion.

Flow cytometry analysis (Figure 3.2) illustrates that the $CD34^+$ cell population (blue area) is located in the correct place in the forward scatter/side scatter (FSC/SSC) distribution and is mostly $CD45^+$ (haematopoietic lineage) as defined by ISHAGE (Sutherland *et al.*, 1996). Before culture, few of the $CD34^+$ cells co-expressed myeloid differentiation markers such CD16b, which is a neutrophil-specific marker. Staining for co-expression of $CD34^+/CD38^+$ the majority of stem cells from mobilised patients expressed CD38. It is known that $CD34^+CD38^-$ cells are more immature stem cells compared to $CD34^+CD38^+$ stem cells (Terstappen *et al.*, 1991; Huang and Terstappen, 1994). Here the initial $CD34^+$ stem cell population was predominantly $CD38^+$ indicating that these cells might be less primitive haematopoietic stem cells.

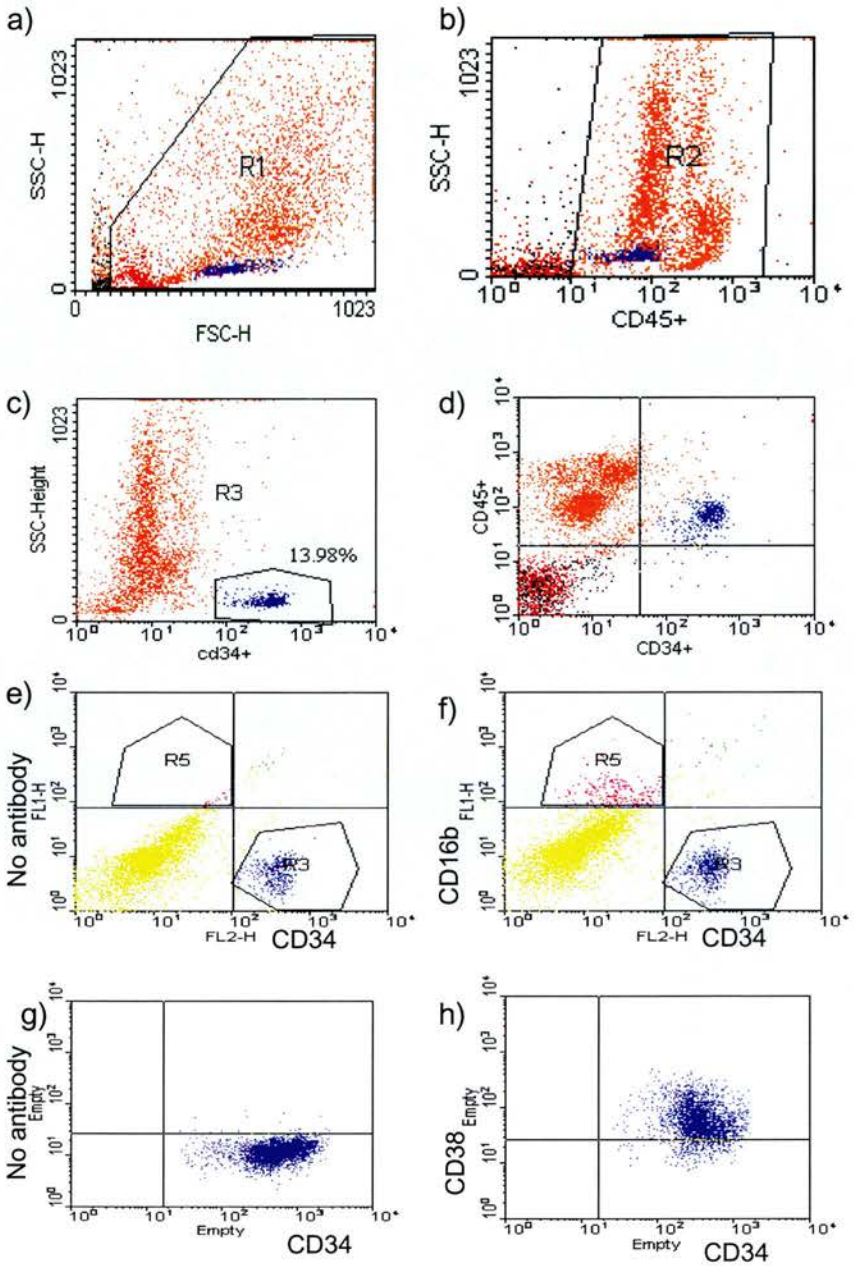


Figure 3.2 Phenotype analysis of the starting population

$CD34^+$ fraction (blue area) in (a) FSC/SSC, (b) SSC/$CD45^+$, (c) SSC/$CD34^+$ and in (d) $CD34^+$/$CD45^+$. CD16b expression: (e) control; $CD34^+$cells without the CD16b antibody and (f) with the presence of the CD16b antibody. None of the $CD34^+$ cells express CD16b. Co-expression CD34/CD38 in (g) control; $CD34^+$ cells without the addition of anti-CD38 and (h) with the presence of CD38 antibody. Most of the cell co-expressed both antibodies. Blood source: G-CSF mobilised peripheral blood.

3.3.3 PBSC expansion

Doses and combinations of cytokines were examined for expansion of $CD34^+$ PBSC in 14-day cultures. Stem Cell Factor (SCF) is a haematopoietic growth factor that exerts its activity by signalling through the c-Kit receptor. SCF and c-Kit are essential for the survival, proliferation and differentiation of haematopoietic cells (Heike *et al.*, 2002). Flt3-Ligand is a growth factor that regulates proliferation of early haematopoietic cells. Flt3-Ligand binds to cells expressing the tyrosine kinase receptor Flt3. Flt3-Ligand, by itself does not stimulate proliferation of early haematopoietic cells, but synergizes with other CSFs and interleukins to induce growth and differentiation (Heike *et al.*, 2002).Initially stem cell factor (SCF) and Flt-3 ligand (Flt3-L) were examined alone and in combination. Concentrations of SCF and Flt3-L (0, 10 and 100 ng/ml) were tested alone or in combination in chequerboard culture experiments (Figure 3.3). SCF and Flt3-L, gave similar expansion cell numbers when used alone, but appeared to synergise when used in combination in that the expansion (more than 20-fold in some cases) exceeded the sum of expansion seen with either alone. There was no significant difference using 10 or 100ng of the Flt3-L in combination with 100ng of SCF ($p=0.25$, Wilcoxon matched pairs test), therefore a combination of 100ng/ml of SCF and 10ng/ml of Flt3-L was selected as optimal for expansion of $CD34^+$ cells over 14 days in culture to conserve Flt3-L use. This mixture of SCF and Flt3-L was referred to as the "expansion cocktail".

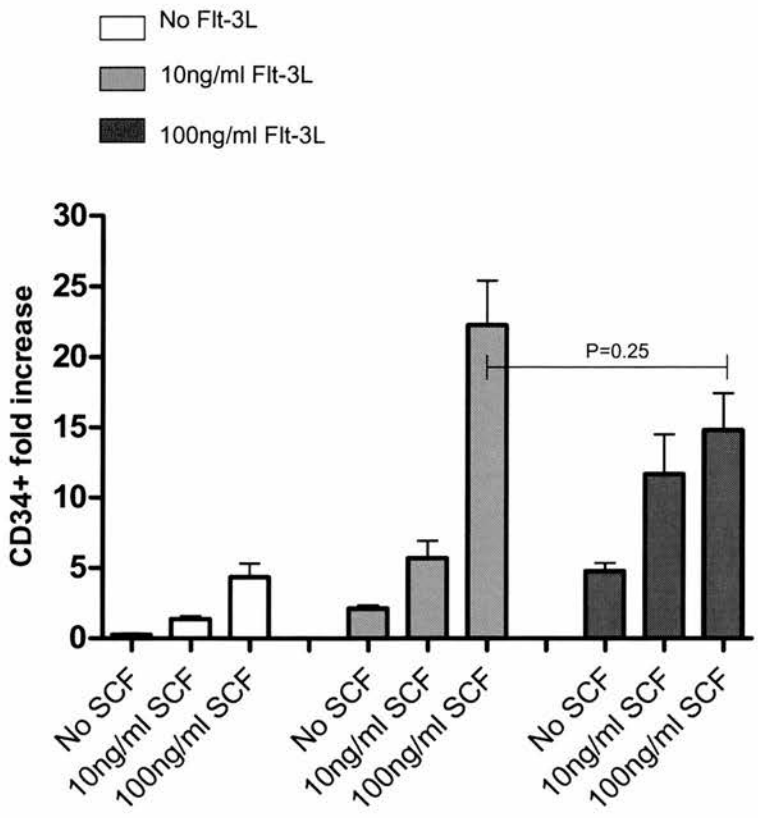


Figure 3.3 Expansion of CD34⁺ cells

Stem cell factor (SCF) and Flt-3 ligand (Flt-3L) in optimal concentrations can expand CD34⁺ cells more than 20-fold in some cases in 14-day cultures (100 ng/ml SCF + 10 ng/ml Flt3-L). Data is presented as mean± SD (n=6 independent experiments). Blood source: G-CSF mobilised peripheral blood.

Figure 3.4 shows the expanded $CD34^+$ cells stained in Wright-Giemsa. The expanded cells are still quite undifferentiated cells with no indication of mature neutrophil morphology. The relative expansion of the cells could be easily seen as increased cell density in culture in phase contrast images (Figure 3.5). The flow cytometry histogram data (Figure 3.6) shows that enriched $CD34^+$ cell population cultured with SCF/Flt3-L for 14 days still retained expression of CD34.

Figure 3.4 Morphology of expanded $CD34^+$ cells

Expanded $CD34^+$ cells after 14-day in cytokine culture conditions stained in Wright-Giemsa. Blood source: G-CSF mobilised peripheral blood.

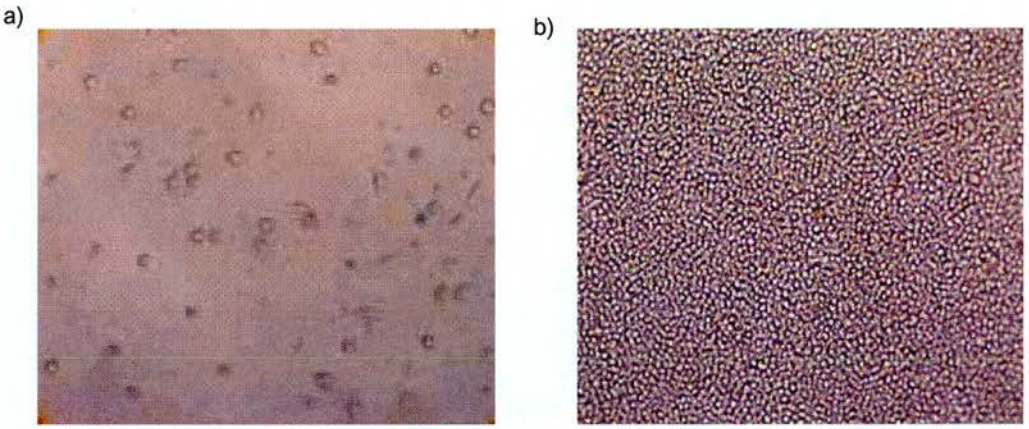


Figure 3.5 Expansion of CD34⁺ cells. Phase-contrast images

Expansion of CD34⁺ enriched cells after 14 days in a 24-well plates (a) without and (b) with SCF/Flt-3L cocktail (phase contrast images). Blood source: G-CSF mobilised peripheral blood.

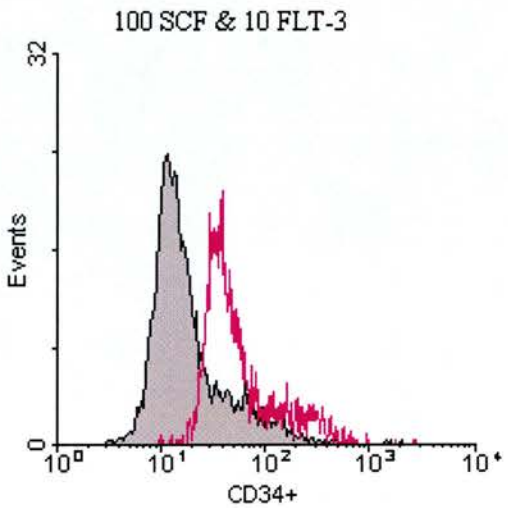


Figure 3.6 CD34 phenotype of the expanded cells

Flow cytometry analysis with (open, purple) and without (shaded, grey) anti-CD34-PE antibody (gated on CD45-PercP bright population). Mostly all of the cells after culture express CD34. Also a brighter CD34⁺ population appears, possibly being a more primitive stem cell population. Blood source: G-CSF mobilised peripheral blood

3.3.4 Addition of other growth factors to improve the previous expansion cocktail.

The addition of other cytokines was examined to determine whether their presence improved expansion of $CD34^+$ cells over that obtained with "expansion cocktail" culture conditions and to assess their effectiveness for differentiation and maturation of the desired cell lineages.

3.3.4.1 Addition of TPO (SCF+Flt3-L+TPO)

TPO is a lineage specific growth factor. It stimulates the proliferation and maturation of megakaryocytes, and promotes increased circulating levels of platelets *in vivo*. TPO signals through the c-mpl receptor and acts as an important regulator of circulating platelets (Heike *et al.*, 2002). It is also been shown to stimulate primitive stem cell expansion and trigger stem cell self-renewal (Heike *et al.*, 2002).In six identical experiments, the addition of TPO (either 10 or 100 ng/ml) to SCF/Flt3-L showed no significant change in cell expansion compared to SCF/Flt3-L alone. SCF/Flt3-L expansion cocktail was still the one preferred for expansion of $CD34^+$ stem cells (Figure 3.7).

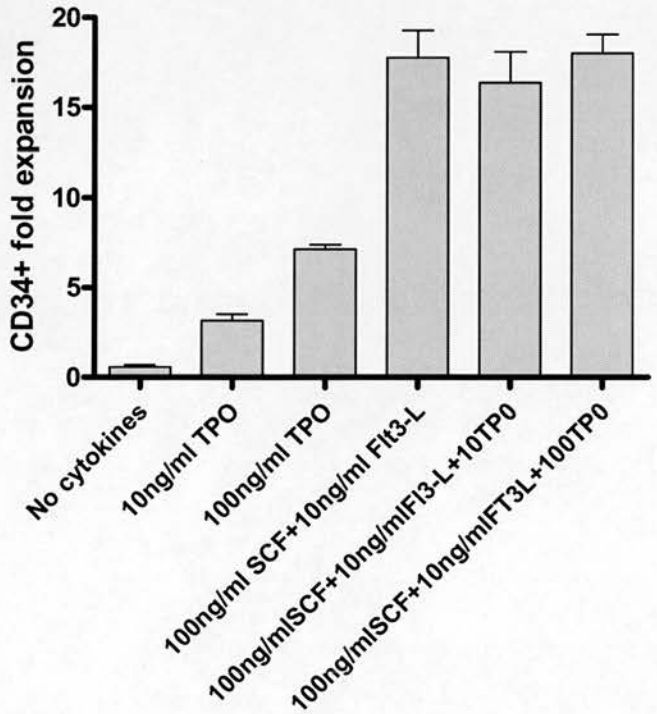


Figure 3.7 Effect of the addition of TPO to the SCF / Flt-3L optimal expansion cocktail

The effect of TPO alone and in combination with "expansion cocktail" (SCF/Flt3-L) on expansion of $CD34^+$ cells in 14-day cultures. Data is presented as mean±SD (n=6 independent experiments). Blood source: G-CSF mobilised peripheral blood.

3.3.4.2 Addition of IL-3 and IL-6

The addition of IL-3 and/or IL-6 to the previous SCF/Flt3-L expansion cocktail was also assessed for improving expansion of $CD34^+$ cells (Figure 3.8). IL-3 is a haematopoietic growth factor that promotes the survival, differentiation and proliferation of committed progenitor cells of the megakaryocyte, granulocyte-macrophage, erythroid, eosinophil, basophil and mast cell lineages (Heike *et al.*, 2002).IL-3 also acts on more immature haematopoietic cells promoting self-renewal (Heike *et al.*, 2002). IL-6 is a pleiotropic cytokine that plays an important role in host defense by regulating immune and inflammatory responses. IL-6 alone does not have distinct biological activity on HSC expansion. IL-6 and IL-3 induce synergistically the proliferation of pluripotent hematopoietic progenitors *in vitro* (Heike *et al.*, 2002). There was no significant change in expansion cell numbers compared to that seen with SCF/Flt3-L alone. For simplicity and to facilitate laboratory procedures in possible GMP clinical application, IL-3 and IL-6 were therefore not considered for addition to the expansion cytokine cocktail.

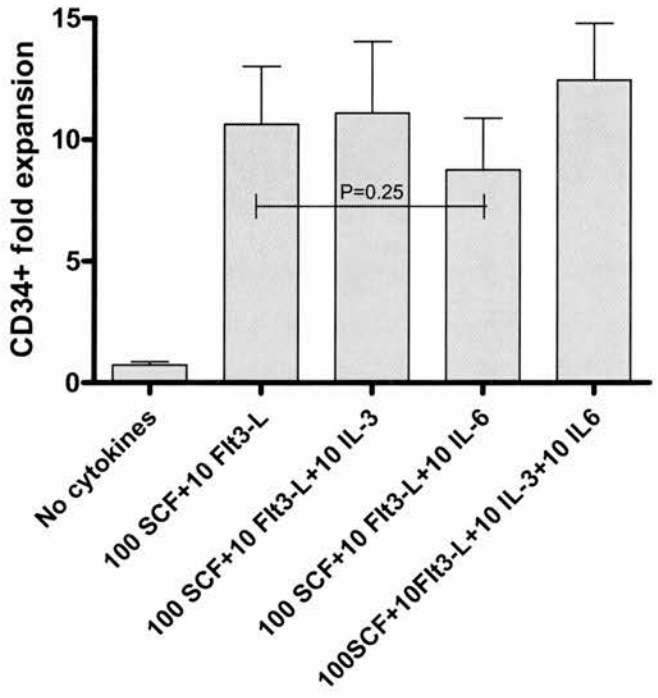


Figure 3.8 Effect of the addition of IL-3 and IL-6 to the SCF/Flt-3L optimal expansion cocktail.
The effect of the addition of IL-3 and IL-6 to the "expansion cocktail" (SCF/Flt3-L) alone or both together on expansion of $CD34^+$ cells in 14-day cultures. Data is presented as mean± SD (n=5 independent experiments). Blood source: G-CSF mobilised peripheral blood.

3.3.4.3 Effect of addition of G-CSF to the SCF/Flt3-L expansion cocktail.

We studied the addition of G-CSF (0, 10 or 100 ng/ml) to the final expansion cocktail (100ng/ml SCF+ 10ng/ml Flt3-L)(Figure 3.9). G-CSF is a haematopoietic growth factor that stimulates the development of committed progenitor cells to neutrophils and enhances the functional activities of the mature end-cell (Dexter, 1994). It is produced in response to specific stimulation by a variety of cells including macrophages, fibroblasts, endothelial cells and bone marrow stroma (Dexter, 1994). Addition of 100ng/ml G-CSF to the SCF/Flt3-L cocktail acted synergistically resulting in expansion of the enriched $CD34^+$ cells (to over 30-fold compared to cells before culture)($p=0.03$, Wilcoxon matched pairs test). Addition of 100ng/ml G-CSF was superior to 10ng/ml G-CSF ($p=0.03$, Wilcoxon matched pairs test). 10ng/ml was not sufficient to stimulate the cells to neutrophil differentiation. The effect of addition of G-CSF to the SCF/Flt3-L combination at the start of the cultures (day 0) was in general greater to than that seen when G-CSF was added midway (day 7) through culture ($p=0.04$, Wilcoxon matched pairs test) and generated cells which showed a more mature myeloid morphology. This implies that G-CSF not only acts to differentiate and mature expanded by SCF/Flt3-L but also synergises with SCF/Flt3-L during early proliferation and expansion.

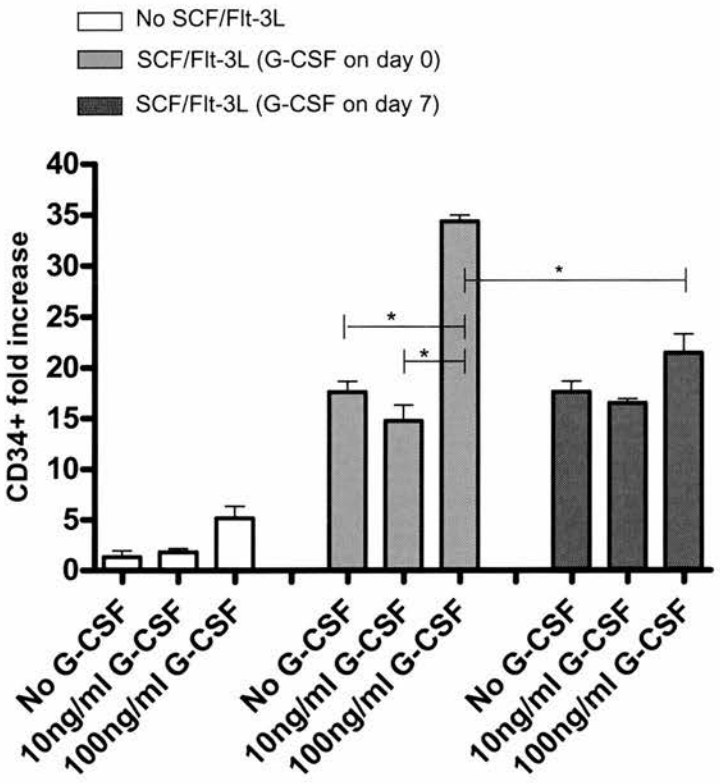


Figure 3.9 Addition of G-CSF to te SCF/Flt-3L optimal expansion cocktail

14-day response to SCF/Flt-3L expansion cocktail with the addition of G-CSF (10 or 100 ng/ml) at day 0 or at day 7. Addition of 100ng/ml G-CSF to the SCF/Flt3-L cocktail acted synergistically resulting in expansion of the enriched $CD34^+$ cells (to over 30-fold compared to cells before culture)($p=0.03$). Addition of 100ng/ml G-CSF was significally superior to 10ng/ml G-CSF ($p=0.03$). The effect of addition of G-CSF to the SCF/Flt3-L combination at the start of the cultures (day 0) was significally greater than that seen when G-CSF was added midway in culture (day 7)($p=0.04$). Data is presented as mean± SD ($n=6$ independent experiments). Arrows indicate significant differences (Wilcoxon matched pairs test). Blood source: G-CSF mobilised peripheral blood.

3.3.5 Phenotypes of expanded cells

After 14 days stimulation with SCF/Flt3-L, all cells were positive for CD34 expression (Figure 3.10c). The majority of cells stained at medium fluorescence intensity, with a small population of CD34-bright cells. When G-CSF was added with SCF/Flt3-L on day 0 (Figure 3.10d) the CD34-bright population disappeared and the fluorescence of the major population (medium fluorescence intensity) had down regulated CD34 expression, becoming dimmer. The addition of G-CSF to SCF/Flt3-L cultures at day 7 (Figure 3.10e) gave similar dimming of the major population medium-fluorescence-intensity CD34 staining-peak was seen through this appeared less marked than when G-CSF was added at day 0. The bright $CD34^+$ population was still present. In no case were there any obvious surviving CD34-negative cells at 14 days, except when G-CSF was used alone without SCF/Flt3-L (Figure 3.10b), which may have prevented apoptosis of myeloid cells.

CD16 was not used in this study since it is not neutrophil specific (positive also in monocytes and NK cells) and our observations indicate that it is labile and rapidly lost from mature neutrophils *in vitro* (data not shown). The expression of CD16b was used to identify neutrophils, as it is a non-labile neutrophil-specific marker. Cells cultured with SCF/Flt3-L+G-CSF showed a small increase in CD16b staining compared to the unstained. However, there was no real difference in CD16b expression between SCF/Flt3-L alone and SCF/Flt3-L+G-CSF (Figure 3.11). The expression was dim in all the cases as compared to mature peripheral blood neutrophils which stain brightly with CD16b. In contrast, there was a major increase in intracyoplasmic myeloperoxidase expression in cells cultured with SCF/Flt3-L/G-CSF compared to cells cultured with SCF/Flt3-L alone (Figure 3.12). Flow cytometry data shows that the expanded cells were relatively positive for the neutrophil CD16b marker and strongly positive for the myeloperoxidase antibody (MPO) demonstrating that the expanded cells were neutrophil precursors.

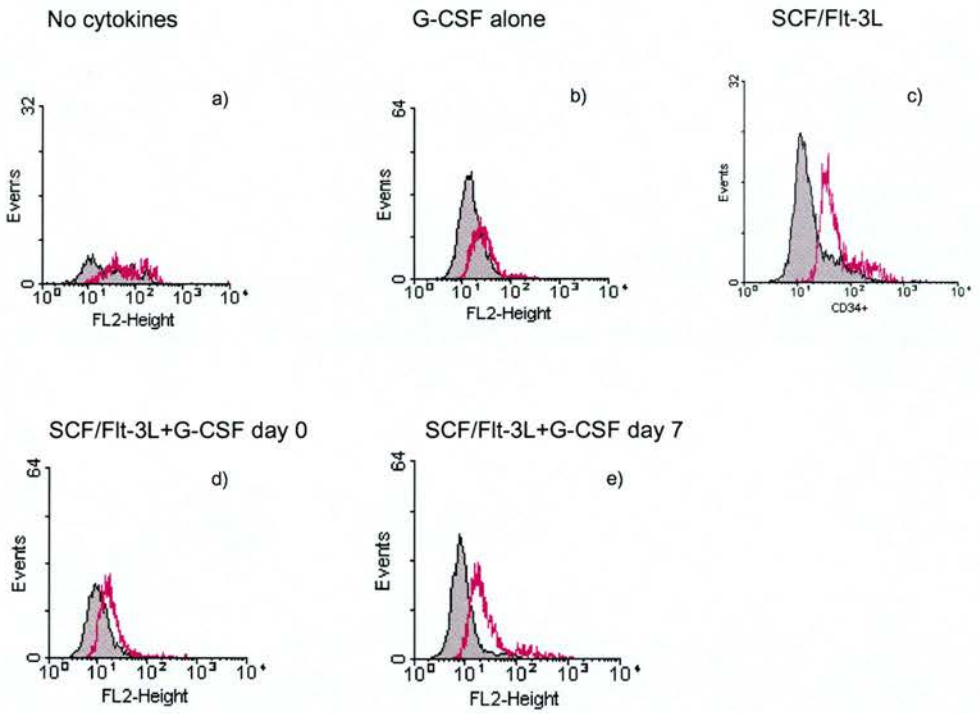


Figure 3.10 Characterisation of expanded CD34⁺ cells with or without the addition of G-CSF.

Flow cytometry analysis with (open, purple) and without (shaded, grey) anti-CD34-PE antibody (gated on CD45-PercP bright population): a) No cytokines, b) G-CSF alone, c) SCF/Flt3L, d) SCF/Flt3L+G-CSF added at day 0 and e) SCF/Flt3L+G-CSF added at day 7 of culture. Cultures with SCF/Flt3L alone retain relatively right expression of CD34 on the expanded cells, whereas when G-CSF is added there is dimming of CD34 expression. Blood source: G-CSF mobilised peripheral blood.

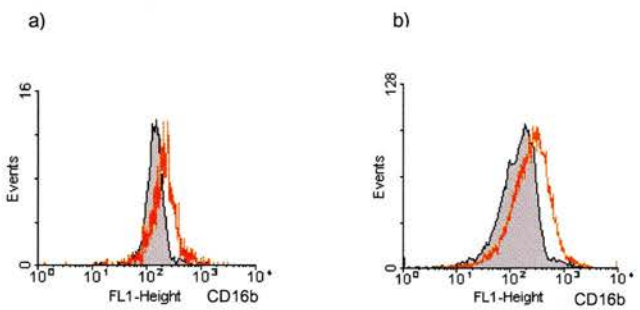


Figure 3.11 Expression of CD16b with or without the addition of G-CSF

Expression of CD16b: Flow cytometry analysis with (shaded grey) and without (open orange) anti-CD16b-FITC antibody (gated on CD45-PercP population); (a SCF/Flt3-L alone and b) SCF/Flt3-L + G-CSF. Blood source: G-CSF mobilised peripheral blood.

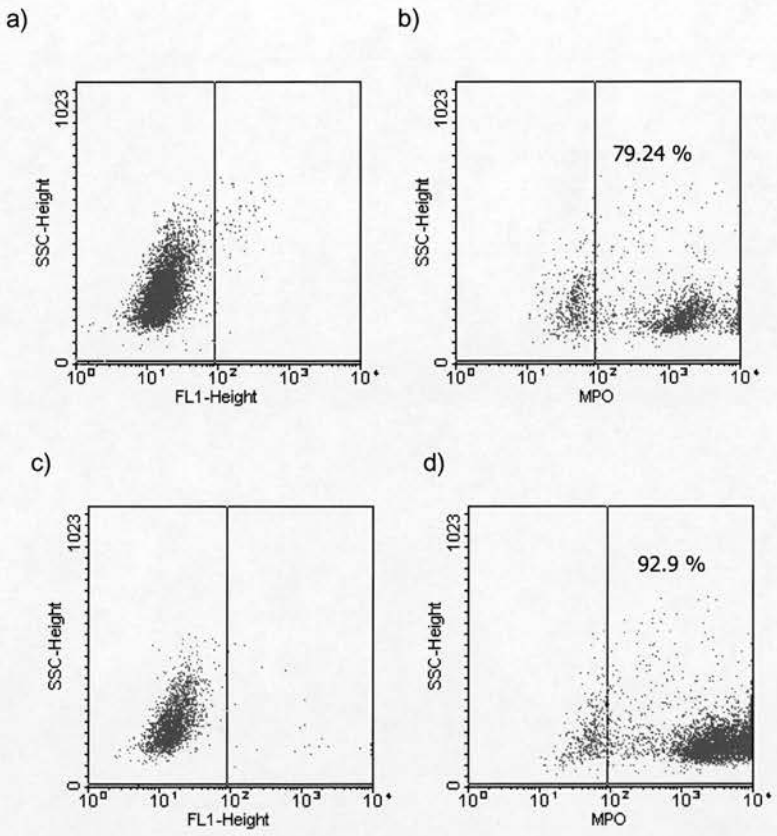


Figure 3.12 Myeloperoxidase expression with or without the addition of G-CSF

Myeloperoxidase (MPO) expression. Flow cytometry analysis (gated on CD45-PercP population); cells cultured in a,b) SCF/Flt3-L alone or c,d) SCF/Flt3-L + G-CSF, a,c) negative control without antibody and b,d) with MPO antibody. Cells cultured in SCF/Flt3-L alone showed less MPO expression compared to cells cultured in SCF/Flt3-L with G-CSF. Blood source: G-CSF mobilised peripheral blood.

3.3.6 Morphology

$CD34^+$ cells expanded with SCF/Flt3-L alone stained with Wright-Giemsa appeared to be morphologically undifferentiated, with no clear indication of development of granulocytic morphology (Figure 3.4). The addition of G-CSF of to cultures gave extensive development of neutrophil morphology. Cells in different stages of neutrophil maturation were found and around 80% of the cells had reached a quite mature stage with apparent segmentation and lobulation of the nuclei and granularity in the cytoplasm (Figure 3.13).

a) b)

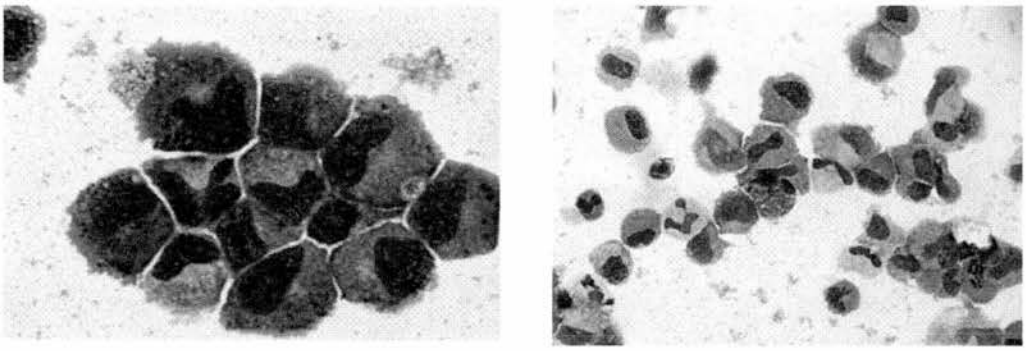


Figure 3.13 Morphology of the cells expanded with G-CSF

These pictures show the expanded $CD34^+$ stem cells after 14-day in cytokine culture conditions stained in Wright-Giemsa. a) SCF/Flt-3L+G-CSF x100 field and (b) SCF/Flt-3L+G-CSF x40field. Blood source: G-CSF mobilised peripheral blood

3.3.7 Effect of the G-CSF mobilising agent

The number of $CD34^+$ cells recovered following culture was compared between G-CSF mobilised samples and cord blood samples, which are not exposed to mobilising or chemotherapy agents *in vivo*, to determine the effect if any of mobilisation with G-CSF on the potential expansion of $CD34^+$ cells. In mobilised blood from patients the expanded cells showed a shift to CD16b expression even without the addition of G-CSF to cultures (expansion cocktail alone) whereas cord blood cells showed little CD16b expression without the addition of G-CSF (Figure 3.14). The addition of G-CSF as a mobilisation agent or even the chemotherapy treatment could have been the cause of this earlier detection in neutrophil expression markers in $CD34^+$ cells from mobilised blood samples.

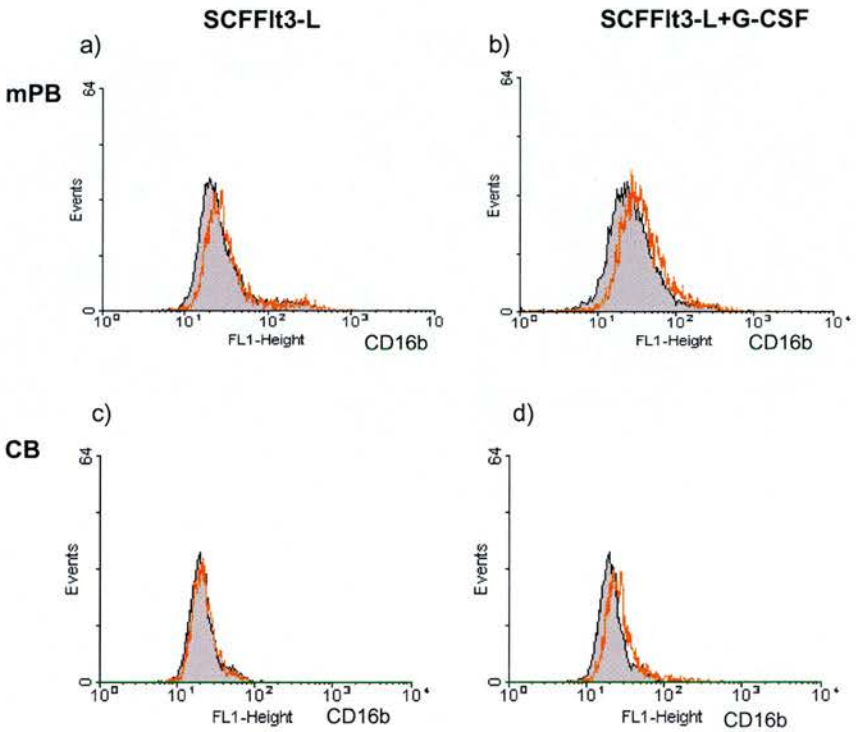


Figure 3.14 Effect of the Mobilisation agents on CD16b expression.

a,c) SCF/Flt3L alone and b,d) SCF/Flt3L+G-CSF; a,b) G-CSF mobilised peripheral blood (mPB) expanded cells and c,d) cord blood (CB) expanded cells. In the G-CSF mobilised patient's blood the cells showed a shift to CD16b expression on expansion even without the presence of G-CSF in culture, whereas cord blood cells showed little CD16b expression without the addition of G-CSF.

3.3.8 Neutrophil superoxide activity.

An important index of neutrophil function is the production of superoxide radicals upon activation. This was measured by detecting luminol-amplified chemiluminescence responses to phorbol myristate acetate (PMA). The respiratory burst activity of peripheral blood neutrophils from healthy adult donors was compared with that of $CD34^+$ enriched mobilised PBSC expanded in SCF/Flt3-L alone or in SCF/Flt3-L+G-CSF (Figure 3.15). $CD34^+$ PBSC cultured with SCF/Flt3-L alone showed no bioluminescence response to PMA, whereas those cultured with SCF/Flt3-L and G-CSF respond like mature healthy neutrophils. Although the starting cell number was the same, the lower response value *in vitro* expanded cells compared to the healthy blood neutrophils was probably because peripheral blood neutrophils are all mature functional cells whereas expanded cells have a difference in functional maturity range.

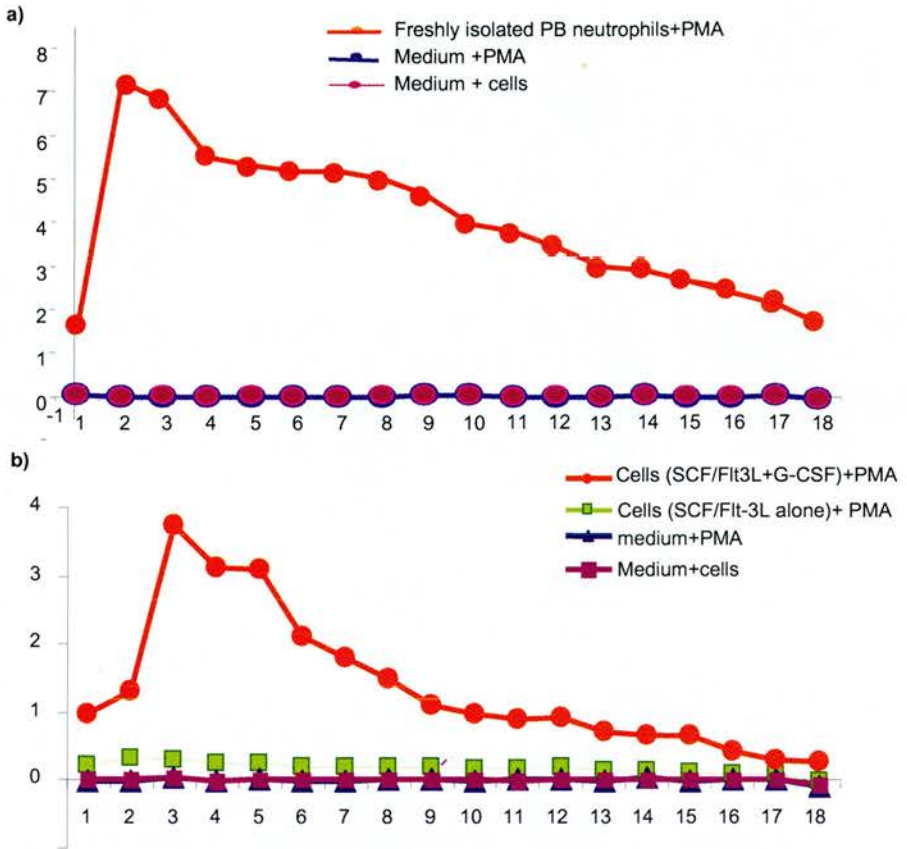


Figure 3.15 Bioluminescence of mature neutrophils and of CD34⁺ cells expanded with or without G-CSF.

Respiratory burst activity of peripheral blood neutrophils from healthy adult donors and that of CD34⁺ enriched mobilised PBSC expanded in SCF/Flt3-L alone or in SCF/Flt3-L+G-CSF. Neutrophils from healthy blood (a) were compared to *ex vivo* expanded CD34⁺ cells (b) Cells cultured in SCF/Flt3-L alone showed no chemiluminescence response, whereas the cells cultured with SCF/Flt3L and G-CSF respond like mature healthy neutrophils.

3.3.9 Duration of cell expansion capacity *in vitro*

To test the period over which expansion could be sustained the initial 14-day culture was extended by additional 14 day periods to 28 and 42 days. At each time point cytokines. Table 3.1 shows CD34⁺ cell expansion after 14, 28 and 42 days. The cells expanded maximally in the first 14-day period but showed only a small further increase in the second 14-day period (to 28 days) and decreased, probably due to cells dying, in the last 14-day period (to 42 days) (Table 3.1). After 28 days of culture

neutrophils were more segmented and mature and after 42 days of culture the cells were blasting and dying (Figure 3.16). Figure 3.17 shows the expression of the neutrophil marker CD16b in cells recovered following culture with SCF/Flt3-L+G-CSF. A brightly staining $CD16b^+$ subpopulation appeared at day 28 which may represent a more mature neutrophil population. Figure 3.16 shows the morphology of these cells at day 28 of culture, which were smaller in size, as compared to day 14, though had more clearly segmented-nuclei characteristic of neutrophils.

CD34+ fold expansion	After 14 days	After 28 days	After 42 days
100 SCF & 10 Flt3-L	15.44	1.53	0.79
100 SCF & 10 Flt3-L +100 G-CSF	19.85	1.6	0.2

Table 3.1 $CD34^+$ fold expansion on extended culture periods.

The cells expand maximally in the first period (14 days) but show only a small increase in the second period (28 days) and start to die in the last period (42 days). Blood source: G-CSF mobilised peripheral blood.

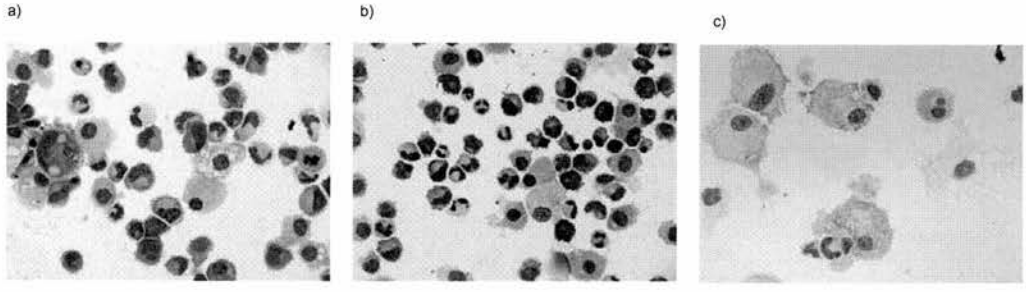


Figure 3.16 Duration of the expansion cell capacity *in vitro*: morphology of surviving cells.

Extension of culture times by further 14 days periods were performed. Cells were harvested, densities adjusted, and placed in fresh cultures after 14 days (to 28 days). This was repeated after a further 14 days (to 42 days). Wright-Giemsa staining a) after 14 days b) After 28 days of culture the neutrophil are more segmented and mature, c) After 42 days of culture the cells are blasting and dying. Blood source: G-CSF mobilised peripheral blood.

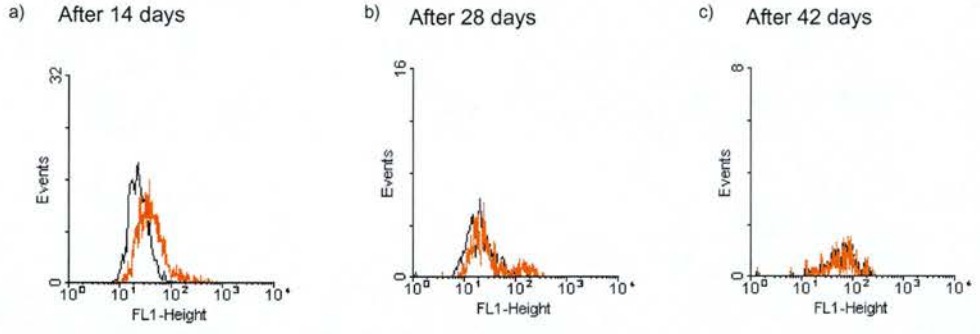


Figure 3.17 The expression of the CD16b⁺ in extended cultures with SCF/Flt3-L+G-CSF after a) 14 days culture, b) 28 days and c) 42 days culture.

A brightly staining CD16b⁺ subpopulation appeared at day 28 which may represent a more mature neutrophil population. At day 42 the cells were blasting and dying and did not express CD16b above background. Blood source: G-CSF mobilised peripheral blood.

3.3.10 Colony assays

Methocult colony assays were carried out for some PBSC blood samples on cells before culture (day 0) and at day 14 following cytokine expansion culture (Table 3.2) to assess their capacity to form colonies for comparison with their behaviour following expansion. To illustrate how colonies were scored typical colonies from the cultures are shown in Figure 3.18. Pie charts (Figure 3.19) show that the potential of the cells before and after expansion is very different. Before expansion, the predominant colonies generated were erythroid BFU-E, but after culture in the presence of SCF/Flt3-L or SCF/Flt3-L+G-CSF cells showed a pronounced shift towards myeloid commitment. There was a marked shift towards CFU-GM myeloid colonies and away from BFU-E in all expanded cultures. CFU-GM were consistently higher and BFU-E lower in cultures with G-CSF compared to SCF/Flt3-L alone, but the switch to CFU-GM with SCF/Flt3-L alone compared to unexpanded cells is almost as large as that with SCF/Flt3-L+G-CSF, and may indicate that most of expanded cells are myeloid committed, even without addition of G-CSF.

	% BFU-E	% CFU-GM+ %CFU-M	BFU-E per thousand cells	CFU-GM per thousand cells	%CD34 in CFC assay
Day 0					
Exp 28	74	26	35.9	12.4	22.7
Exp 29	74	26	8.6	3	62.33
Exp 32	72	28	5	1.9	16.56
Day 14					
Exp 28 SF	10	90	3	28.5	100
Exp 28 SFG	5	95	0.8	16.1	100
Exp 29 SF	18	82	1.1	4.9	100
Exp 29 SFG	9	91	0.4	4.5	100
Exp 32 SF	13	87	0.67	4.7	100
Exp 32 SFG	0	100	0	4.7	100

Table 3.2 Methocult colony capacity before and after cell expansion

Colony forming cell (CFC) comparisons in 3 sets of mobilised PBSC before culture (day 0: $CD34^+$ enriched cells) and after culture (day 14: expanded $CD34^+$ enriched cells) with SCF/Flt3-L (SF) and with SCF/Flt3-L+G-CSF (SFG). The proportional (percent) results were used for qualitative comparisons. Some indication of quantitative responses can be obtained from CFC numbers, which may relate to $CD34^+$ numbers allowing for $CD34^+$ expansion.

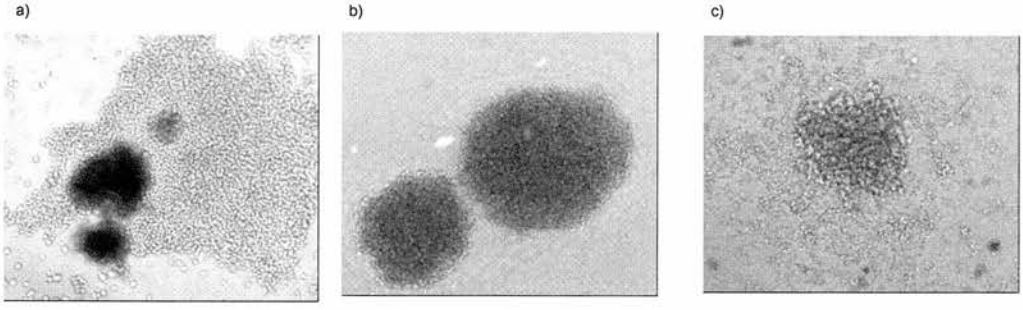


Figure 3.18 Methylcellulose colony scoring

Colonies in Methylcellulose. Phase contrast images (X100 field) a) CFU-GEMM, b) BFU-E, c) CFU-GM. Blood source: G-CSF mobilised peripheral blood

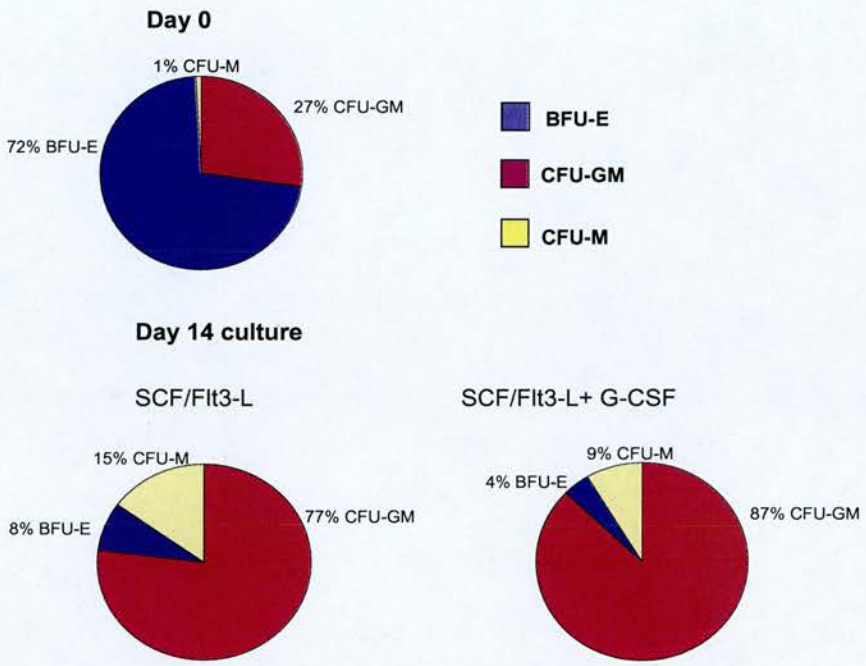


Figure 3.19 Methylcellulose colony assay: frequencies of colony types before and after culture with cytokines.

Before culture (day 0) the predominant colonies generated were BFU-E, but after culture with SCF/Flt3-L+G-CSF (day 14) a major generation of myeloid CFU-GM colonies was observed. Blood source: G-CSF mobilised peripheral blood

3.3.11 Do CD34 negative cells contribute to the observed CD34⁺ expansion?

Because the CD34⁺ enriched samples were not completely CD34⁺ pure, the contribution of the remaining CD34-negative cell population after positive enrichment of CD34⁺ cells was assessed. Using the same culture conditions as previously described both CD34-negative and CD34⁺ fractions were cultured with the addition of the expansion cocktail (SCF/Flt3-L) with or without G-CSF. As shown in Figure 3.20 the CD34-negative fraction showed no cell expansion in the presence of any cocktail of cytokines used. Thus, only the CD34⁺ cells were responsible for cell expansion after 14-days. Moreover, before culture CD34-negative cells did not form colonies in methylcellulose (data not shown). Thus, any remaining CD34-negative cells such as monocytes or lymphocytes did not contribute to the cell-expansion seen after 14 days in culture. However, it is not known whether their presence in the culture is beneficial for expansion of CD34⁺ cells as they may secrete positive or negative growth factors.

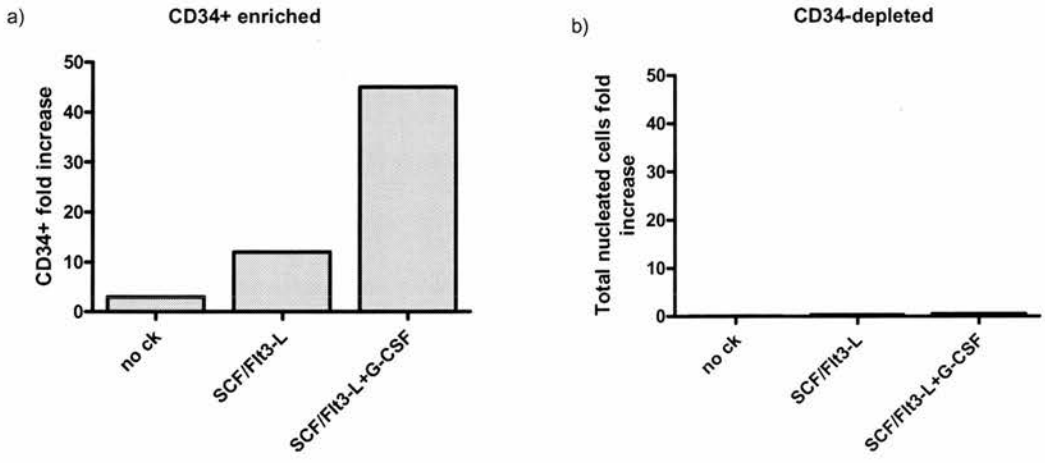


Figure 3.20 Examination of whether CD34-depleted MNC contribute to cell expansion

a) The $CD34^+$ isolated from MACS had a fold expansion of 45 when cultured with SCF/Flt-3L+G-CSF in this experiment b) The CD34-negative population did not show any expansion at all after 14 day of cytokine culture. Blood source: G-CSF mobilised peripheral blood

3.3.12 Comparison of the expansion/maturation cocktail (SCF/ Flt3-L ± G-CSF) with cytokine combinations described in the literature.

Recently, many investigators have published their optimal PBSC expansion cocktails. Clinical studies carried out by McNiece *et al.* have used the following cocktail of cytokines: 100ng/ml of (SCF+TPO+G-CSF). Our "expansion cocktail" defined here (100ng/ml SCF+ 10ng/ml Flt3-L+ 100ng/ml G-CSF) was compared to McNiece *et al.* cytokine cocktail and no significant improvement was found both in terms of cell expansion (Figure 3.21) and differentiation (3.22). Piciabello *et al.* used a complex cytokine combination with the addition of IL-3 and IL-6. The addition of either Il-3 or IL-6 alone or together slightly decreased expansion compared to optimal SCF/ Flt-3-L/G-CSF expansion. Changes were not significant both in terms of cell expansion and differentiation (Figure 3.23a and 3.24). Since these effects were not incremental, and for the sake of simplicity for any implementation of clinical protocols for cell expansion, it was decided that inclusion of IL-3 and IL-6 gave no apparent advantage, and that the SCF/Flt-3-L/G-CSF cocktail would be used for sufficient generation of neutrophil progenitors.

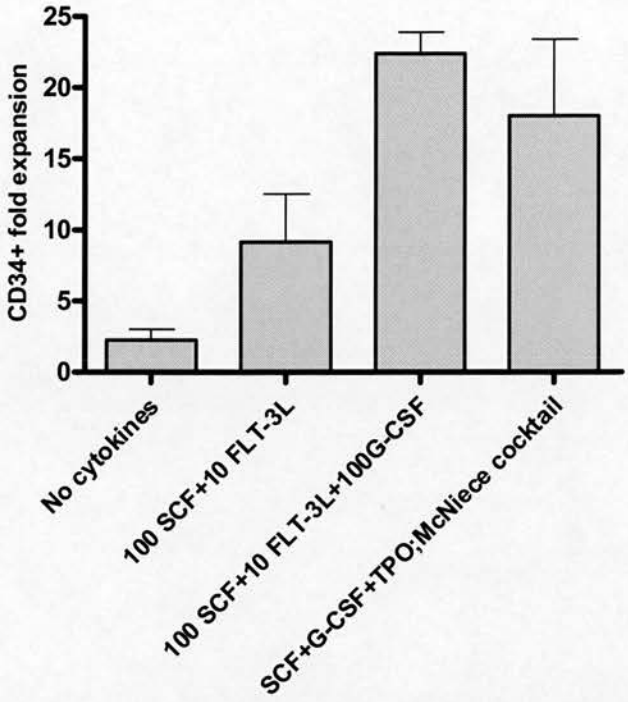


Figure 3.21 Comparison of cell expansion between our SCF/Flt3-L±G-CSF optimal cocktail with that published by McNiece *et al.*.

Our shown "expansion cocktail" ± G-CSF defined here (100ng/ml SCF+10ng/ml Flt3-L±100ng/ml G-CSF) is compared to McNiece *et al.* cytokine cocktail (100ng/ml SCF+100ng/ml G-CSF+100ng/ml TPO). Data is presented as mean± SD (n=3 independent experiments). Blood source: G-CSF mobilised peripheral blood

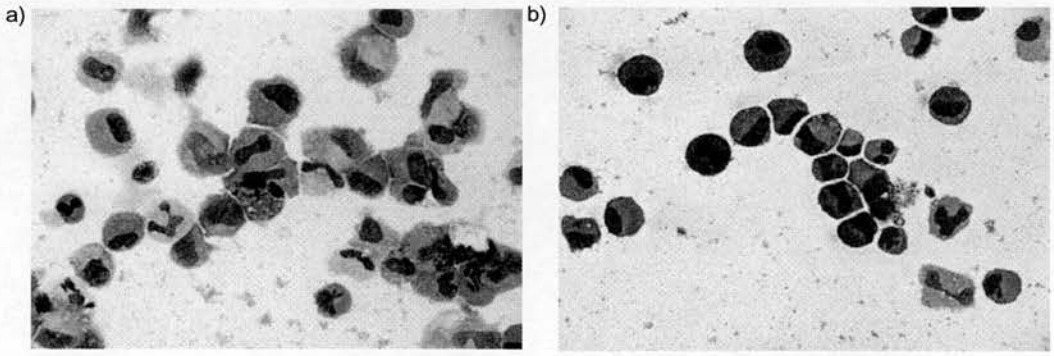


Figure 3.22 Comparison of neutrophil maturation between our SCF/Flt-3L+G-CSF optimal cocktail with that published by McNiece *et al.*

a)"Expansion cocktail" +G-CSF defined here (100ng/ml SCF+10ng/ml Flt-3L+100ng/ml G-CSF) and b) McNiece *et al* cytokine cocktail (100ng/ml SCF+100ng/ml G-CSF+100ng/ml TPO). Our combination of cytokines appear to have better neutrophil maturation. Blood source: G-CSF mobilised peripheral blood

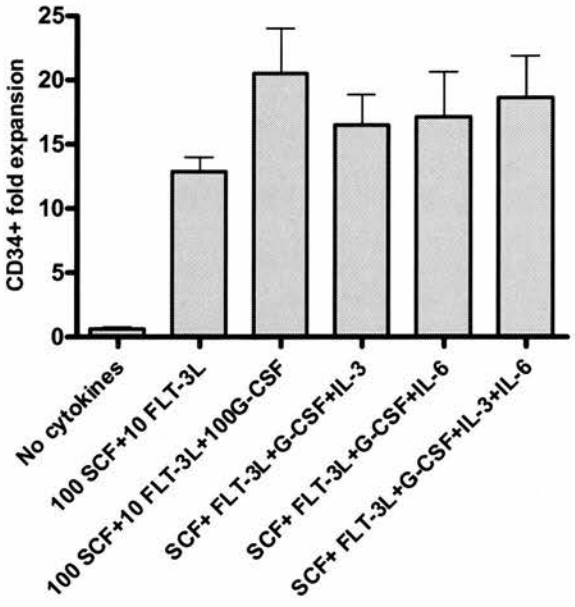


Figure 3.23 Comparison of expansion between our SCF/Flt-3L±G-CSF optimal cocktail with that published Piciabello *et al.*

Our "expansion cocktail" ±G-CSF defined here (100ng/ml SCF+ 10ng/ml Flt-3L ±100ng/ml G-CSF) was compared to Piciabello *et al* cytokine cocktail (100ng/ml SCF+10ng/ml Flt-3L+100ng/ml G-CSF ±10ng/ml IL-3 ± 10ng/ml IL-6). Data is presented as mean± SD (n=3 independent experiments). Blood source: G-CSF mobilised peripheral blood

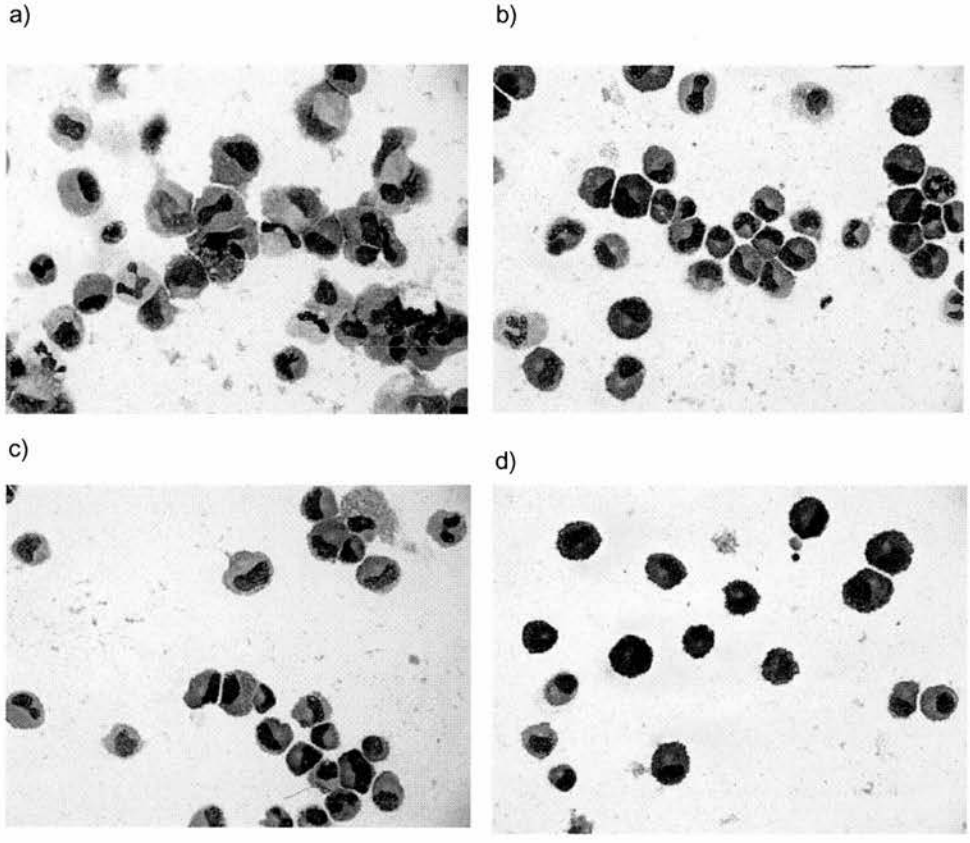


Figure 3.24 Comparison of maturation of neutrophils between our SCF/Flt-3L+G-CSF optimal cocktail with that published Piciabello *et al*.

a)"Expansion cocktail"+ G-CSF (100ng/ml SCF+10ng/ml Flt-3L+100ng/ml G-CSF)

b) 100 SCF+10 Flt3-L+ 100 G-CSF+ 10 IL-3, c) 100SCF+10Flt3-L+100G-CSF+10 IL-6 and

d) 100 SCF+10 Flt3-L+100 G-CSF+10 IL-3+ 10 IL-6. Expansion cocktail"+ G-CSF appears to be the best combination of cytokines for neutrophil differentiation. Blood source: G-CSF mobilised peripheral blood.

3.3.13 Effects of TPO on optimal expansion and maturation of CD34$^+$ enriched mobilised PBSC.

The effect of addition of TPO to the SCF/Flt3-L/G-CSF neutrophil expansion/ differentiation cocktail was tested by examining cell expansion, morphology and function. The addition of TPO to SCF/Flt3-L showed no significant change in expansion compared to SCF/Flt3-L alone as described above (3.3.4.1). The addition of TPO to SCF/Flt3-L/G-CSF resulted in a slight reduction or had no effect ($p = 0.0625$, Wilcoxon matched pairs test, n=5) on the expansion achieved with SCF/ Flt3-L/G-CSF alone (Figure 3.25). Certainly TPO offered no advantage over SCF/ Flt3-L/G-CSF alone in terms of cell expansion, and could be omitted for the sake of simplicity on these grounds alone. Cells expressing CD41 were found in small numbers in cultures to which TPO was added, but were not found in any cultures where TPO was omitted (Figure 3.26). When cells were counted and scored according to their degree of neutrophil differentiation, cells stimulated with SCF/ Flt3-L/G-CSF+ TPO were less differentiated with a less mature neutrophil morphology than cells cultured with SCF/Flt3-L/G-CSF alone (Figure 3.27). Myeloperoxidase (MPO) activity is found in mature neutrophils and myelocyte progenitors. The CD34$^+$ cells cultured with SCF/Flt3-L/G-CSF and TPO showed a marginal decline in intensity of expression of MPO compared with the cells cultured without TPO (Figure 3.28). However, since MPO stains most of the stages of the committed neutrophil differentiation pathway then an effect of TPO on the stage of neutrophil maturation achieved might not be clearly detected by this method. Neutrophil superoxide activity is a good indicator of neutrophil functional maturity. CD34$^+$ cells cultured with SCF/Flt3-L/G-CSF showed a maximal superoxide response to PMA comparable to fresh mature peripheral blood neutrophils (3.3.8). CD34$^+$ cells cultured with SCF/Flt3-L without G-CSF showed little superoxide response whether or not they were cultured with TPO. When TPO was added to CD34$^+$ cells cultured with SCF/Flt3-L/G-CSF, the superoxide response was reduced, but not abolished (Figure 3.29). In this respect, TPO appears antagonistic to the effect of G-CSF on promoting maturation of neutrophil superoxide activity in the expanded CD34$^+$ cells.

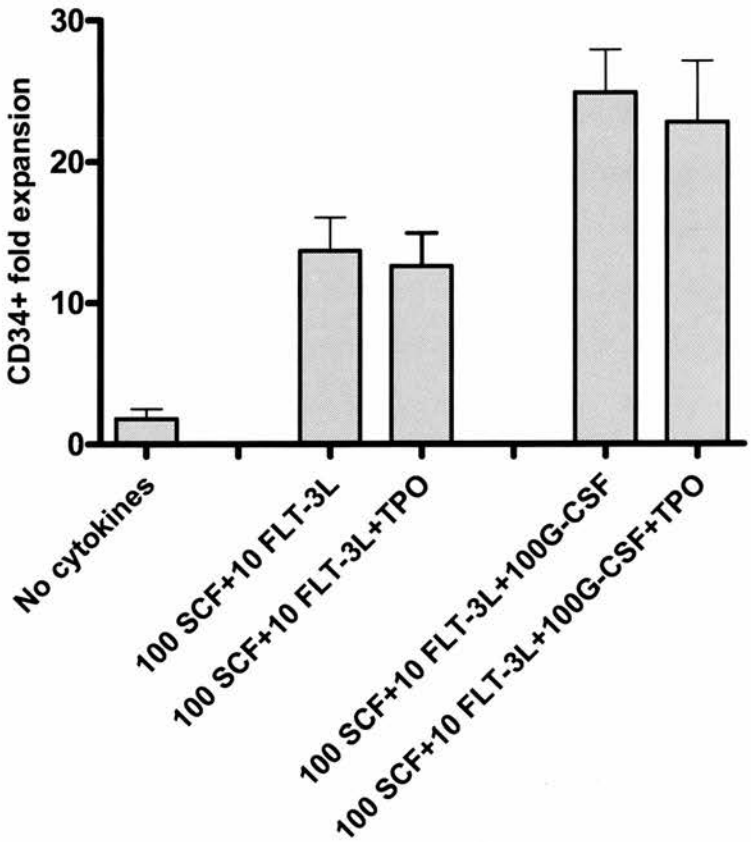


Figure 3.25 Effect of the addition of TPO on SCF/Flt3-L+G-CSF cell expansion.

Our "expansion cocktail" ± G-CSF defined here (100ng/ml SCF+10ng/ml Flt-3L± 100ng/ml G-CSF) was compared to the addition of TPO (100ng/ml SCF+10ng/ml Flt-3L ± 100ng/ml G-CSF +100 ng/ml TPO). Data is presented as mean± SD (n=5 independent experiments). Blood source: G-CSF mobilised peripheral blood.

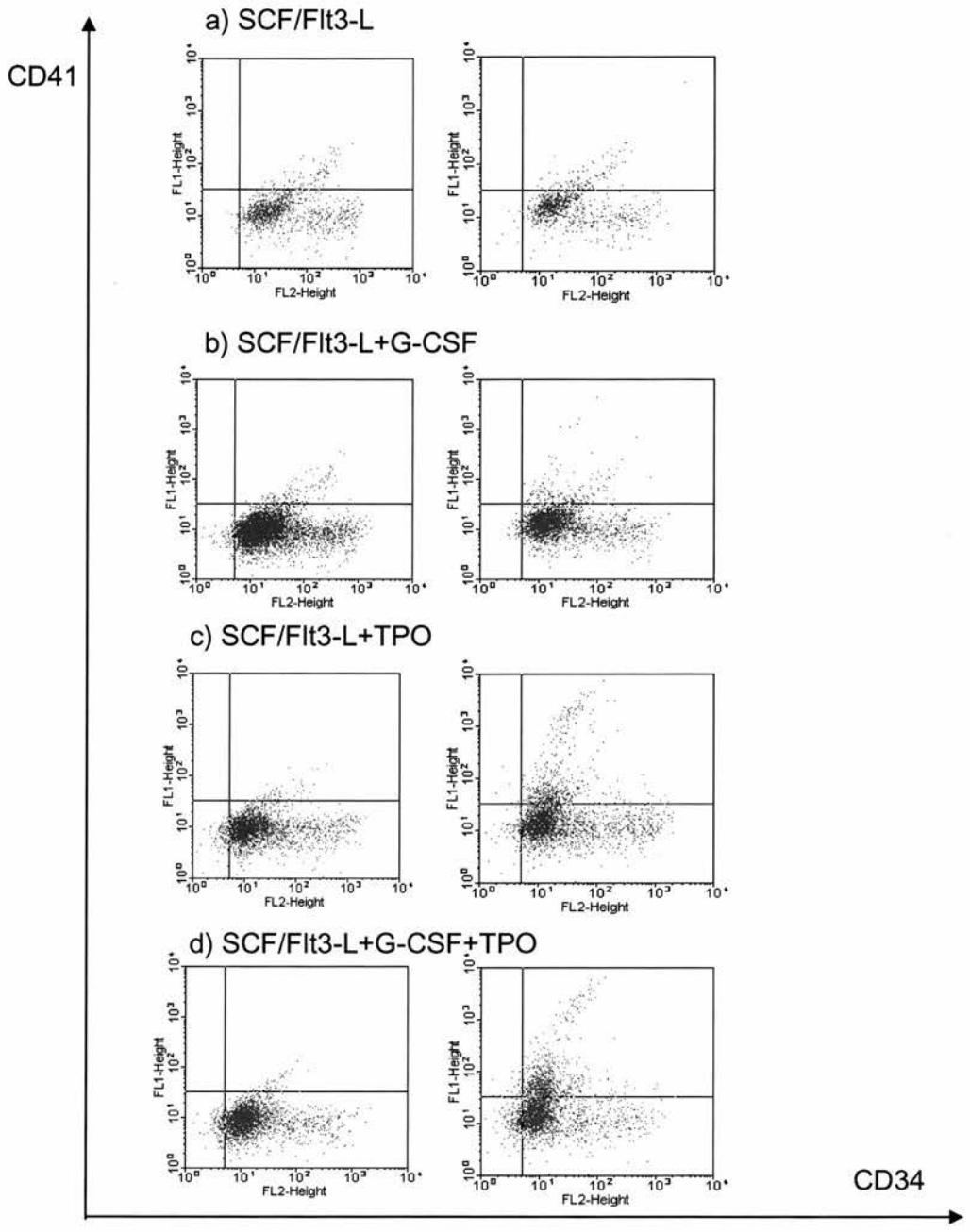


Figure 3.26 Megakaryocyte differentiation in expansion cultures with or without TPO

The expression of the CD41 platelet marker in cultures with or without TPO. Left column control without CD41 antibody and right column with the presence of the antibody. a) SCF/Flt3-L alone, b) SCF/Flt3-L+G-CSF, c) SCF/Flt3-L+TPO and d) SCF/Flt3-L+G-CSF+TPO. Blood source: G-CSF mobilised peripheral blood.

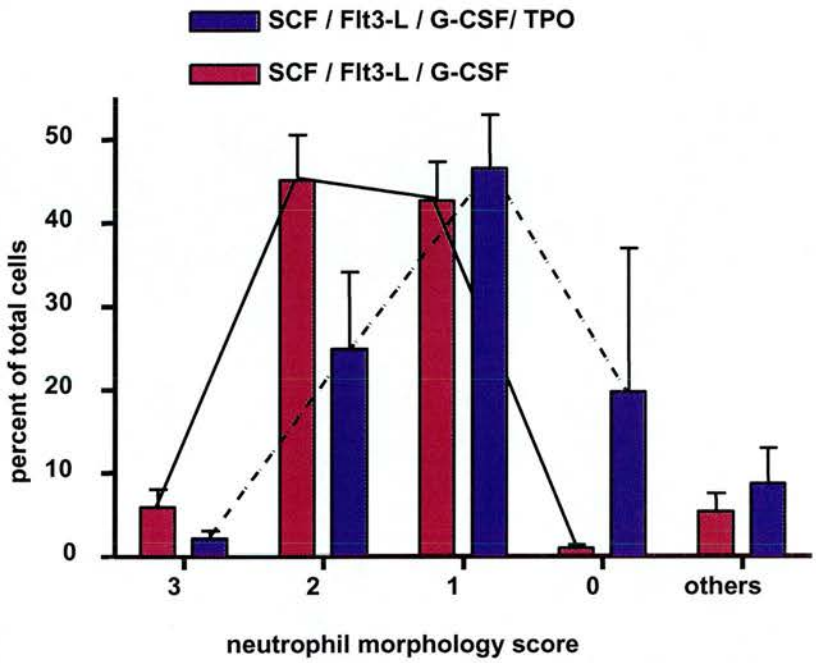


Figure 3.27 Effect of the addition of TPO on neutrophil maturation morphology in expansion cultures.

Cells cultured with SCF/Flt3-L/G-CSF + TPO showed a delay in neutrophil differentiation by morphology compared to cells cultured without TPO (SCF/Flt3-L/G-CSF alone). Scoring was determined by assessing neutrophil morphology (see methods) ranging from 0 (no differentiation) to 3 (most mature). Others represent cells which had no neutrophil or stem cell morphology. Blood source: G-CSF mobilised peripheral blood.

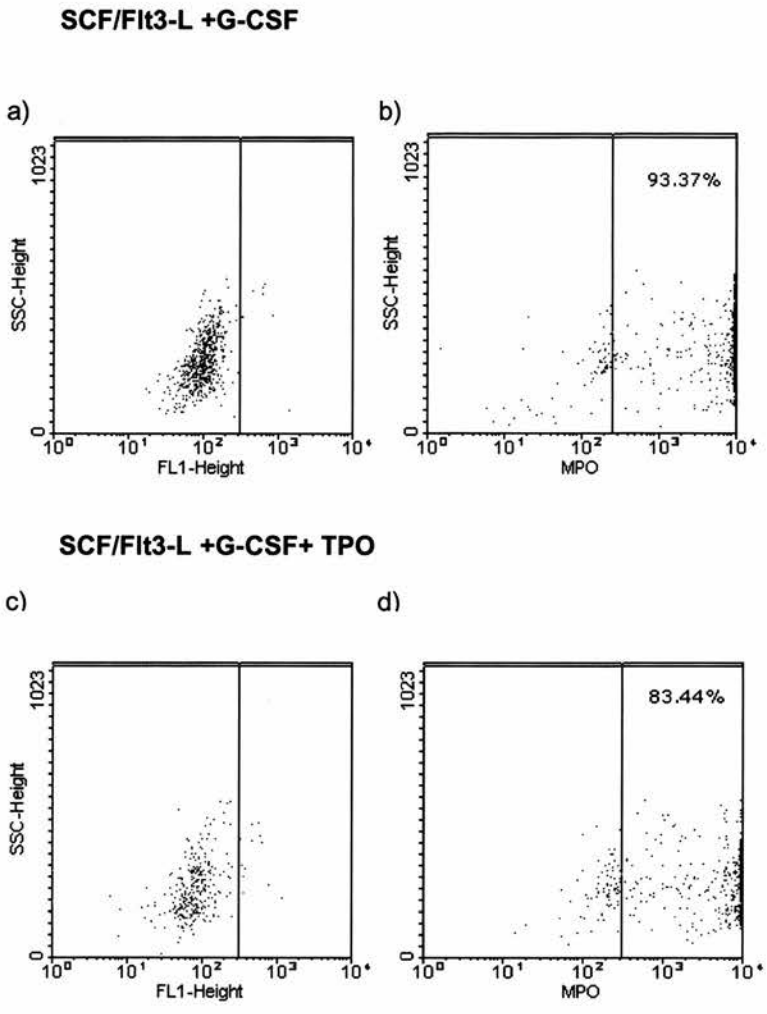


Figure 3.28 Effect of TPO on MPO expression in cells undergoing ex vivo neutrophil expansion
a,b) SCF/Flt3-L+G-CSF and c,d) SCF/Flt3-L+G-CSF+TPO; a,c) negative controls without antibody and b,d) with MPO antibody. Cells cultured with SCF/Flt3-L/G-CSF+ TPO showed a decline in intensity of expression of MPO(83.44%) compared with the cells cultured without TPO(93.37%). Blood source: G-CSF mobilised peripheral blood.

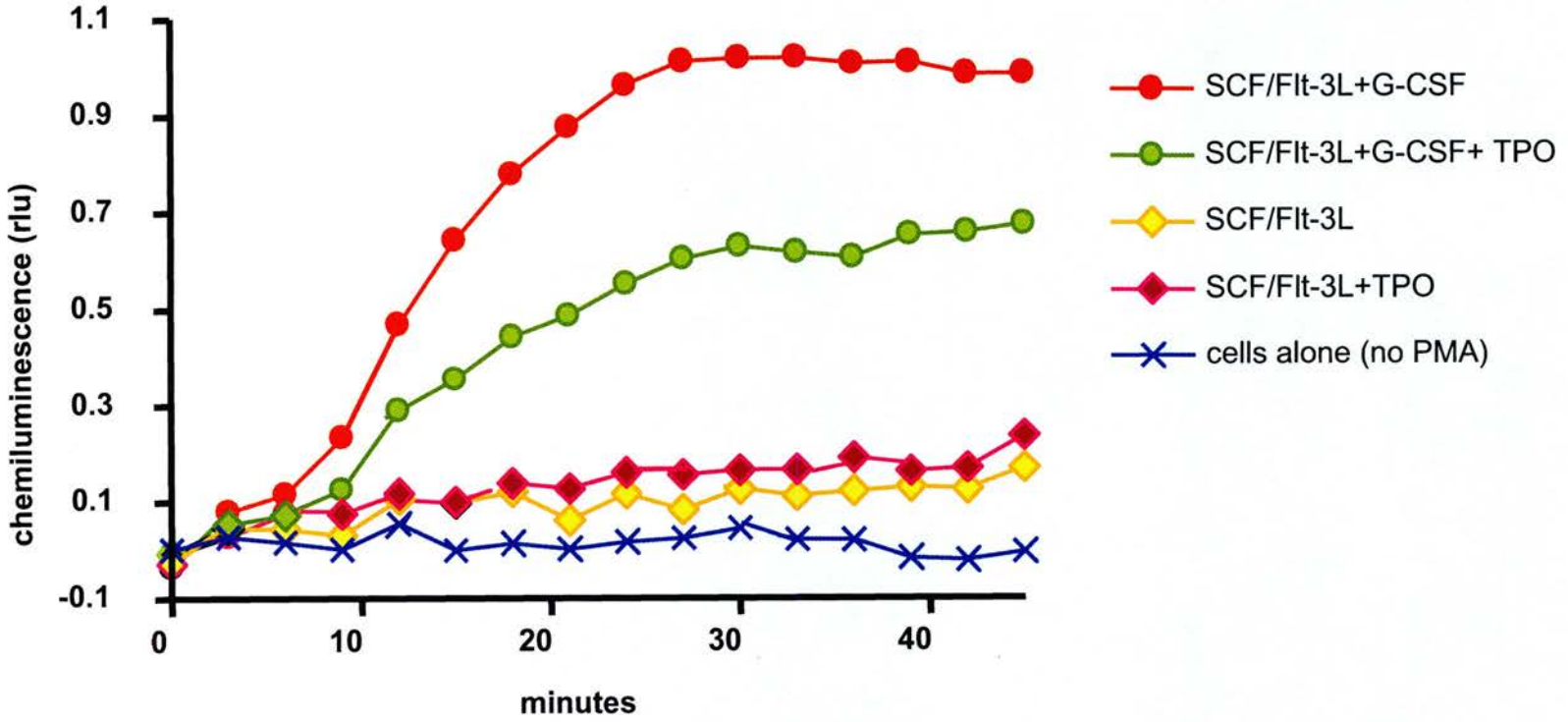


Figure 3.29 Effect of the addition of TPO on superoxide response of cells expanded with SCF/ Flt3-L±G-CSF.

Cells cultured with SCF/Flt-3L+G-CSF showed maximal superoxide response to PMA. When TPO is added to this, the response is reduced. Cells cultured without G-CSF showed little response to PMA. Blood source: G-CSF mobilised peripheral blood.

3.4 Discussion

Here it has been demonstrated that a $CD34^+$ cell population enriched from whole mobilised peripheral blood either from patients or healthy G-CSF mobilised donors can be optimally expanded in culture for 14 days using 100ng/ml SCF+10ng/ml Flt3-L combination of growth factors. SCF and Flt3-L synergised to provide considerable proliferative stimulus to expand PBSC $CD34^+$ cells in culture. This expansion is entirely derived from the $CD34^+$ population, as CD34-depleted population has been shown not to contribute to this growth. Addition of G-CSF to SCF and Flt3-L further expanded total PBSC $CD34^+$ cells and differentiated and matured them towards the neutrophil lineage. The resultant cells down-regulated the expression of CD34 compared to SCF/Flt3-L alone and acquired neutrophil morphology and function as assessed by myeloperoxidase activity and superoxide generation in response to PMA. Expression of CD16b by expanded cells was dim compared to mature neutrophils and differences in CD16b expression between SCF/Flt3-L and SCF/Flt3-L/G-CSF expanded cells were marginal. Intracytoplasmic myeloperoxidase activity (MPO) appears in earlier stages of myeloid differentiation than CD16b expression therefore, MPO was a more useful marker in terms of the study of its expression in early-mid stages of neutrophil cell maturity. Addition of G-CSF to SCF/Flt3-L was required for major expression of myeloperoxidase activity and essential for superoxide production. Bioluminescence assay of neutrophil function showed that only the cells cultured in the presence of SCF/Flt3-L+G-CSF were mature enough to respond like mature healthy neutrophils. In this study colony assays showed slight differences between cells expanded with SCF/Flt3-L and cells expanded with SCF/Flt3-L/G-CSF. More BFU-E colonies were retained in the absence of G-CSF, but myeloid colonies predominated in both expansion cultures, whereas erythroid colonies clearly predominated before *ex vivo* expansion, in agreement with results reported by Reichle *et al.*, 2003. Thus colony assays did not sensitively assess the effects of the addition of other cytokines to SCF/Flt3-L.
These results are comparable with early preclinical studies reported on *ex vivo* expansion of PBSC $CD34^+$ even where more complex cytokine combinations were used to accelerate neutrophil maturation *in vitro* (Haylock *et al.*, 1992; Brugger *et al.* 1993). No significant increase of significant cell numbers or neutrophil differentiation was detected in here by supplementary addition of IL-3, IL-6 or TPO, alone or in combination. Flt3-L might not have been available

to these investigators, and it appears that it may substitute for other cytokines previously used in expansion cocktails, synergising with SCF to induce HSC expansion. This indicates that for selective *ex vivo* expansion of neutrophil precursors from PBSC, it is sufficient to use Flt3-L with SCF and G-CSF for maximal expansion, and that supplementary inclusion of TPO, IL-3 and/or IL-6 used by other authors is superfluous and increases the complexity and cost of the protocol. This early preclinical studies (Haylock *et al.*, 1992) promoted expansion of autologous $CD34^+$ PBSC to the myeloid lineage while retaining part of the harvest unmanipulated. Alternatively, many studies have been trying to achieve simultaneous expansion of committed progenitors and preservation or expansion of more primitive multipotent HSC in the same system. Holyoake *et al.*, 1997 demonstrated clinically that transplantation solely with *ex vivo* expanded $CD34^+$ cells does not confer durable haematopoietic reconstitution. Similar results have been found using cord blood. As has recently been reviewed (Robinson *et al.*, 2005) the goal of many cord blood expansion protocols remains a single complex expansion system delivering mature erythroid, myeloid and megakaryocyte precursors for rapid reconstitution with retention of multipotent stem cells for durable reconstitution. The lack of durable reconstitution by expanded cord blood cells alone has been confirmed in animal studies (McNiece *et al.*, 2002), and indicates the necessity of ensuring retention of multipotent long-term reconstituting HSC, for which single system protocols are still being sought (Peled *et al.*, 2004). Some cord blood studies therefore propose the use of multiple cords for a single transplant, some of which may be manipulated *ex vivo* for selective lineage expansion for more rapid resolution of neutropenia (Pecora *et al.*, 2000; Shpall *et al.*, 2002; Jaroscak *et al.*, 2003). It was not possible nor was the aim of this study to maintain an immature expanded $CD34^+$ cells population. After culture all $CD34^+$ PBSC were in some degree of neutrophil differentiation. In a clinical perspective to ensure long-term reconstitution an unmanipulated part of the harvest would be given as an adjunct to the differentiated cells. This study has shown that the primary wave of $CD34^+$ stem cell expansion occurred in the first 14 days of culture (proliferative phase). After 14-days in culture the cells lost proliferative potential having probably already become committed cells. During further 14-days (28 days) of culture the cells appeared to advance their maturation process. This point is important to consider for future clinical practice, suggesting that $CD34^+$ cells fully matured to neutrophils *in vitro* may have little further proliferative potential, and therefore may not expand further *in vivo*. Any infusion would therefore have to deliver an adequate number of cells directly and could not rely on these being supplemented in the short term by proliferation of manipulated

precursors *in vivo*. However it may be possible to manipulate the cells to a less mature, but more proliferative point in their development, which could be more optimal for clinical use, providing both early and adequate neutrophil recovery from manipulated cells until long term recovery from the unmanipulated PBSC graft takes over. This would depend on the clinical purpose. More mature functional neutrophils ready to work as soon as they are injected or less differentiated neutrophils with retained proliferative capacity could be recovered before or after 14 days of cultured and infused when the patient requires them. Further investigation is required before safe and effective clinical application is possible. Cells may require storage before use, if so, should they be manipulated after freezing since neutrophils do not survive freezing. Also the timings of harvest, manipulation and engraftment need to be addressed. In addition it will be necessary to the procedure to apply GMP standards.

Cord blood samples were also used in comparison with G-CSF mobilised peripheral blood and *ex vivo* neutrophil differentiation was equally achieved with no noticeable differences. In some G-CSF mobilised samples even in the absence of G-CSF in culture (that is SCF/Flt3L alone), the $CD34^+$ expanded cells shifted towards the myeloid phenotype whereas cord blood samples did not. This was probably due to the preceding clinical mobilisation process where the cells were exposed to G-CSF *in vivo*, thus SCF/Flt3-L alone was able to push the cells along the differentiation pathway to which prior exposure to G-CSF may have made them susceptible.

A small number of studies have investigated selective *ex vivo* expansion exclusively of neutrophil progenitors from PBSC (Reichle *et al.* 2003; Hino *et al.*, 2000; Scheding *et al.*, 2000; Zimmerman *et al.*, 2000) and in two such studies the expanded cells have been used clinically to supplement autologous PBSC. Results were inconclusive in one clinical study (Zimmerman *et al.*, 2000) but reduced neutropenia in the other (Reichle *et al.* 2003). Most of the e*x vivo* protocols and preclinical trials, have aimed to achieve simultaneous expansion of both neutrophil and megakaryocytes precursors to address the dual problems of neutropenia and thrombocytopenia (McNiece *et al.*, 2000; Paquette *et al.*, 2000; Prince *et al.*, 2004; Reiffers *et al.*, 1999). Therefore addition of TPO, favoured in protocols aimed to produce simultaneous expansion of both neutrophil and megakaryocyte precursors was examined. As presented above combining the $CD34^+$ cell expansion cocktail (SCF/Flt3-L) with the platelet progenitor cytokine (TPO) gave no benefits in terms of cell expansion. Cells expressing CD41 (megakaryocyte marker) were found in small numbers only in cultures to which TPO was added. Thus, it appears that the addition of TPO is essential to megakaryocyte (platelet) formation, but a synergistic stimulus from

other cytokines seems to be required as we did not achieved major platelet differentiation in any of our cultures. On the other hand, supplementary addition of TPO to SCF/Flt3-L/G-CSF resulted in a retardation of neutrophil maturation in both morphology and function. MPO expression was reduced when TPO was added to the SCF/Flt3-L/G-CSF combination, and less differentiated neutrophils also resulted in cultures with the presence of TPO. These results are consistent with the findings of others that TPO is not necessary for selective neutrophil expansion from PBSC (Reichle *et al.* 2003; Hino *et al.*, 2000; Scheding *et al.*, 2000; Zimmerman *et al.*, 2000) or cord blood (De Bruyn *et al.*, 2003). Its inhibitory effect on neutrophil maturation is probably marginal, but it is certainly superfluous for induction of specific neutrophil precursor expansion. No obvious advantage in megakaryocyte production was detected either, therefore its addition to the SCF/Flt3-L/G-CSF cytokine combination is unfounded. Consistent with the *in vitro* observations reported here, it has been reported that *in vivo* administration of TPO delays myeloid recovery following chemotherapy (Geissler *et al.*, 2003). Others have also suggested that selective megakaryocyte expansion may be best achieved with quite different cytokine combinations from those required for neutrophil precursor expansion (Bertolini *et al.*, 1997). More complex combined expansion protocols do not appear to have been evaluated clinically. Selective *ex vivo* expansion of megakaryocyte precursors by relatively simple protocols including SCF, TPO and IL-3 (Scheding *et al.*, 2004), or SCF and TPO only (Decaudin *et al.*, 2004), showed no clinical benefit for thrombocytopenia reduction when expanded cells were administered as an adjunct to unmanipulated autologous PBSC compared with that achieved using unmanipulated autologous PBSC. The reported clinical effect on reduction of thrombocytopenia ranged from no effect (McNiece *et al.*, 2000; Reiffers *et al.*, 1999) to a reduction in post-transplant platelet transfusion requirement (Prince *et al.*, 2004) or a mean reduction in thrombocytopenia duration by 1 day (Paquette *et al.*, 2000). It therefore appears that so far, clinical thrombocytopenia, associated with autologous PBSC transplantation is not amenable to reduction by simple cytokine *ex vivo* expansion protocols.

From the data presented here it would appear that the use of two separate protocols might be beneficial. One to expand/differentiate neutrophil precursors (SCF/Flt3L/G-CSF) and the other to generate megakaryocyte progenitor cells. These two different cell populations appear to follow quite separate differentiation pathways in the haematopoietic maturation system, and very probably may require completely different stimuli and culture conditions to generate each cell type. PBSC expansion protocols all employ SCF as the major direct proliferative stimulus for

primitive and committed HSC. Other cytokines are used with SCF to provide additional direct stimuli, or synergistic stimuli, for proliferative expansion. Cytokines are employed to stimulate mature and immature HSC to differentiate and mature along specific lineage-committed progenitor pathways, and may also provide proliferative and survival signals for these committed cells. It may be naïve to assume that all of these processes can procede simultaneously in the same system without competition or inhibition within different HSC expansion and differentiation pathways. It may be best clinically to focus on selective neutrophil precursor expansion as a feasible and needed goal for reduction of neutropenia, and if megakaryocyte generation is to be considered then separate protocols should be devised to optimise this in distinct cultures rather than attempting simultaneous neutrophil and megakaryocyte generation in the same system. Hence, while support for thrombocytopaenia is routinely available from transfusion centres through allogeneic leukodepleted platelet transfusions, which present low risk of virus transmission (van de Watering, 2004), there are only few centres offering routine granulocyte transfusion. Granulocyte donations can only be stored for hours and the logistical difficulties in recruiting histocompatible and virus-screened donors are substantial. Moreover, ethical considerations regarding mobilisation of granulocytes in such donors have not been fully assessed, nor the efficacy of granulocyte transfusion for treatment of neutropenia (Briones *et al.*, 2003; Price, 1998; Strauss, 1999; Yeghen and Devereux, 2001).

In summary, the results presented here suggest that the combination of SCF, Flt3-L and G-CSF enables $CD34^+$ HSCs to differentiate into functional neutrophils and should be investigated for the development of protocols suitable for clinical scale-up and compliance, as a possible cost-effective means of reducing neutropenia following autologous PBSC transplant which is not addressed by availability of donor granulocyte transfusion. Further studies are required to investigate the generation of functional megakaryocytes precursors but extensive literature reports suggest that this remains unfeasible so far. These results could be also used as a $CD34^+$ HSC differentiation model where expression of different genes and proteins could be studied along their differentiation pathway. In the next chapter the expression of different phosphatases is studied while $CD34^+$ HSCs differentiated towards the neutrophil lineage.

Chapter 4

Control of neutrophil survival and function.

(A study in collaboration with Isabelle V. W. M. Heinisch, Centre for Inflammation Research, University of Edinburgh Medical School, QMRI, Edinburgh, UK)

4.1 Introduction

A collaborative study was undertaken to combine *ex vivo* expansion and differentiation of $CD34^+$ cells to neutrophils with investigation of the role of tyrosine phosphatases SHP-1 and SHP-2 in regulating apoptosis in neutrophils. I provided neutrophils at different time points of maturation as well as immunophenotype analysis, measurement of cell death after culture, transfection of $CD34^+$ differentiated cells and cell morphology by Giemsa staining. Dr. Isabelle Heinisch conducted the western blotting separations. Protocols were modified and adjustments of cell culture times were discussed in accordance with the results obtained.

Neutrophil granulocytes play a key role in the innate immune system protecting the host from invading bacteria. Neutrophils are produced in great number in the bone marrow, and released into the blood stream. Once a neutrophil is released into circulation and tissue, it remains there only a few hours. In the absence of activating stimuli provided by invading bacteria, neutrophils undergo spontaneous apoptosis (programmed cell death) and are cleared by phagocytes. This cellular homeostasis must be tightly regulated to provide an effective host defence and to avoid self-damage.

Apoptosis seems to be critically involved in the control of elimination of cells within the body and thus contributes to the maintenance neutrophil turnover in the circulation, helping to keep a balance between cell proliferation and cell death (Weinmann *et al.*, 2003). A dysregulation of neutrophil apoptosis may contribute

to the expansion or the reduction in the number of peripheral neutrophils in the pathogenesis of leukaemia and infections (Weinmann *et al.*, 2003).

Tyrosine phosphorylation, controlled by the coordinated action of protein tyrosine phosphatases (PTPs) and kinases (PTKs), is a critical control mechanism for numerous physiological processes, including growth and differentiation, cell survival and death (Tonks and Neel, 1996). Tyrosine phosphatases containing SH2 domains (SHPs) have been identified in a variety of species but only two SHPs exist in higher organisms; SHP-1 and SHP-2.

SHP-1 is expressed at highest level in haematopoietic cells and in a murine model of SHP-1 deficiency mice displayed a range of haematopoietic abnormalities (Tonks and Neel, 1996). SHP-2 is expressed ubiquitously and a recent study has identified mutation in human SHP-2 as the cause of the inherited disorder Noonan syndrome and might also contribute for the pathogenesis of some leukaemias (Neel *et al.*, 2003). Moreover, Chan *et al.*, (2003) showed that SHP-2 function is critical for the maintenance of a proper balance of ES cell differentiation, pluripotency and apoptosis.

Phosphatase and tensin homologue (PTEN) is one the most frequently mutated tumour suppressor in human cancer and it is also essential for embryonic development. Functions of PTEN have been identified in the regulation of many normal cell processes including growth and apoptosis (Yamada and Araki, 2001; Di Cristofano and Pandolfi, 2000).

Pathways regulated by the two SHPs have been specified and several potential targets identified. Remarkably, despite their shared domain structure and considerable (55%) overall sequence identity, the SHPs appear to have different roles. Early studies indicated that SHP-1 was predominantly a negative regulator of PTK signalling, whereas SHP-2 played a positive (i.e. signal-enhancing) role (Tonks and Neel, 1996).

To get a better understanding of the role of tyrosine phosphatases involved in the regulation of neutrophil survival and cell death we investigated the expression of the SHP-1, SHP-2 and PTEN in *ex vivo* cultured $CD34^+$ haematopoietic stem cells. Our working hypothesis is that either a balance between the inhibitory tyrosine phosphatase SHP-1 and the activating tyrosine phosphatase SHP-2, or the equilibrium between phosphatases and kinases might regulate the commitment of neutrophils to cell death before well-defined apoptotic changes occur (I.Heinisch *et al.*, unpublished data). Furthermore, we assume that SHP-1 regulates spontaneous neutrophil cell death, and SHP-2 prolongs neutrophil survival and the balance of SHP-1 and SHP-2 determines neutrophil cell fate. SHP-2 is very weakly or not

expressed in healthy peripheral blood neutrophils, and expression could not be induced by the survival factor GM-CSF (I.Heinisch *et al.*, unpublished data). Furthermore, we showed (I.Heinisch *et al.*, unpublished data) that in two leukaemic cell lines K562 and KG-1 an inverse SHP-1/SHP-2 balance in favour of SHP-2, can be found, which might explain the failure of cell death in these cells. The purpose of this collaboration is to evaluate the use of our *ex vivo* CD34-derived neutrophils as an *in vitro* differentiation model to further test this hypothesis, and to follow up the expression and function of the phosphatases SHP-1 and SHP-2 and PTEN on neutrophil differentiation.

4.2 Experimental procedure

$CD34^+$ cells were cultured in the presence of SCF/Flt-3L for 7, 14, 21 day with or without the addition of G-CSF. Whole cell lysates were collected at each time point and SHP-1, SHP-2 and PTEN expression studied by Western blotting. As demonstrated in Chapter 3, $CD34^+$ cells cultured for 14 days in the presence of G-CSF resemble functionally mature neutrophils in many respects while in the absence of G-CSF the expanded $CD34^+$ cells expressed few mature neutrophil functions. Cells were collected at different stages of the differentiation process and were also transfected with a SHP-1-GFP (and control GFP) construct in order to investigate the effect of SHP-1 on the life span of these cells. The SHP-1-GFP plasmid was a kind gift from Paul Crocker.

4.3 Results

4.3.1 SHP-1 expression

Two forms of SHP-1 (one of 62kDa and 70kDa) were detected. Neutrophils isolated from healthy adults as well as *in vitro* differentiated neutrophils from $CD34^+$ haematopoietic cells cultured for 14–days expressed a 62kDa and 70kDa form of SHP-1. In contrast, the promyelocytic leukaemic HL-60 cells only expressed the 70kDa form or no SHP-1 at all (Figure 4.1) (Heinisch, I *et al.*, manuscript in preparation).

Healthy neutrophils expressed higher amounts of the 62kDa SHP-1 band than 70kDa SHP-1 band (Figure 4.1a) whereas *ex vivo* expanded $CD34^+$ cells expressed higher amounts of the 70kDa than the 62kDa SHP-1 (Figure 4.1b). There was no difference in SHP-1 expression between cells cultured for 14 days in the presence or the absence of G-CSF (Figure 4.1).

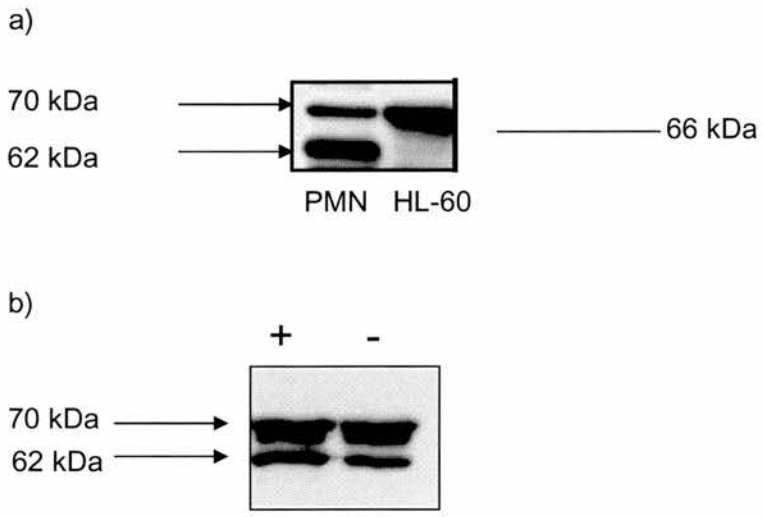


Figure 4.1 SHP-1 expression in peripheral blood neutrophils, HL-60 cell line and G-CSF-stimulated $CD34^+$ cells at 14 days *ex vivo*.

SHP-1 expression in a) healthy neutrophils (PMN) and HL-60 (promyelocytic leukaemic cell line) b) *ex vivo* differentiated neutrophils from $CD34^+$ cells after 14 days culture with SCF/Flt3-L and with (+) or without (-) G-CSF.

To follow SHP-1 protein expression during the *in vitro* differentiation process $CD34^+$ cells were analysed in terms of SHP-1 expression at day 7, 14 and 21 days. There is a reduction of the 70kDa SHP-1 expression as the cells differentiate towards a more mature neutrophil cell population with almost no expression at day 21. There also seems to be a reduction of 62KDa SHP-1 band with its disappearance at day 21 (Figure 4.2). There was not a significant difference in expression between cells cultured in SCF/FLt3-L alone or with the addition of G-CSF or TPO at any time point analysed. However, this reduction of SHP-1 expression seems to be slightly enhanced by the addition of G-CSF (Figure 4.2).

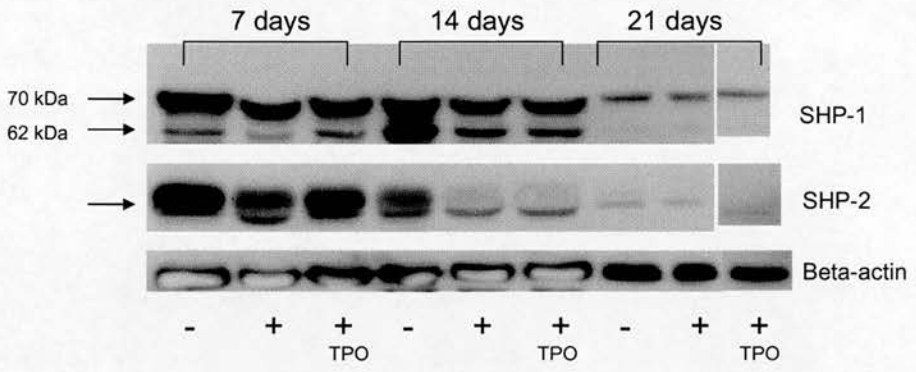


Figure 4.2 SHP-1 expression in G-CSF-stimulated $CD34^+$ cells at 7 day intervals.

Ex vivo differentiated neutrophils from $CD34^+$ cells after 7, 14 and 21 days culture with G-CSF(+), without it (-) or with G-CSF and TPO (+ TPO) or without TPO (-TPO)(all cultures included SCF/Flt3-L). At day 0 and 7 of the culture where more immature cells were found, the 70kDa band is much stronger compared to the 62kDa band. As long as the cells mature the upper 70kDa band got smaller. Similarly, as the cells differentiate towards a more mature neutrophil cell population the SHP-2 band got slighter. Beta actin is added as a loading control.

Extended *in vitro* $CD34^+$ differentiation for up to 21 days resulted in 93.8% apoptosis and protein analysis at this time point was not possible anymore.

Increase of culture period resulted in an increase of mature neutrophils in culture and correlated with an increase of Annexin-V staining (Figure 4.3). At day 7 of culture, with or without G-CSF, most of the cells retained high proliferative capacity and low expression of Annexin-V (19.84% SCF/Flt-3L alone compared to 29.77% (SCF/Flt-3L+G-CSF). At day 14 of culture more functional differentiated neutrophils are found with the presence of SCF/Flt-3L+G-CSF compared to with SCF/Flt-3L alone (see also Chapter 3). Thus some mature neutrophils cultured with SCF/Flt-

3L+G-CSF started to apoptose showing a higher expression of Annexin-V (50.19%) compared to SCF/Flt-3L alone (26%). At day 21 almost all the cells cultured in the presence of SCF/Flt-3L+G-CSF were apoptotic (93.80%) compared to cells cultured with SCF/Flt-3L alone (45.11%). Double staining with propidium iodide (PI) confirmed that these cells die due to apoptosis and not because of necrosis. The morphology of cultured cells at the different time points are shown in Figure 4.4. Cells at day 7 had an undifferentiated morphology in both SCF/Flt-3L alone or SCF/Flt-3L+G-CSF. The addition of G-CSF increased expression of granulocytic cytoplasm and the frequency of segmented nuclei at day 14 compared with cells cultured with SCF/Flt-3L alone, and at day 21 of culture cells started to blast and die.

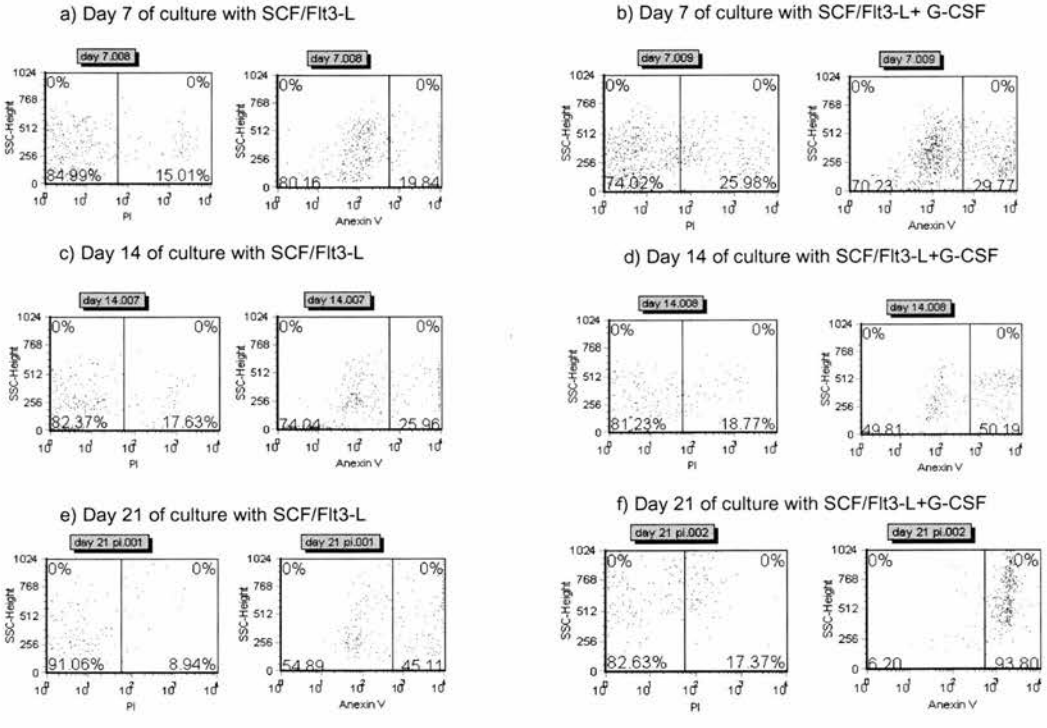


Figure 4.3 Apoptosis and cell death in CD34⁺ cells at 7 day intervals with/without G-CSF.

Measurement of cell death after 7, 14, 21 days of culture with SCF/Flt-3L with or without G-CSF by PI uptake and Annexin-V staining determined by flow cytometry.

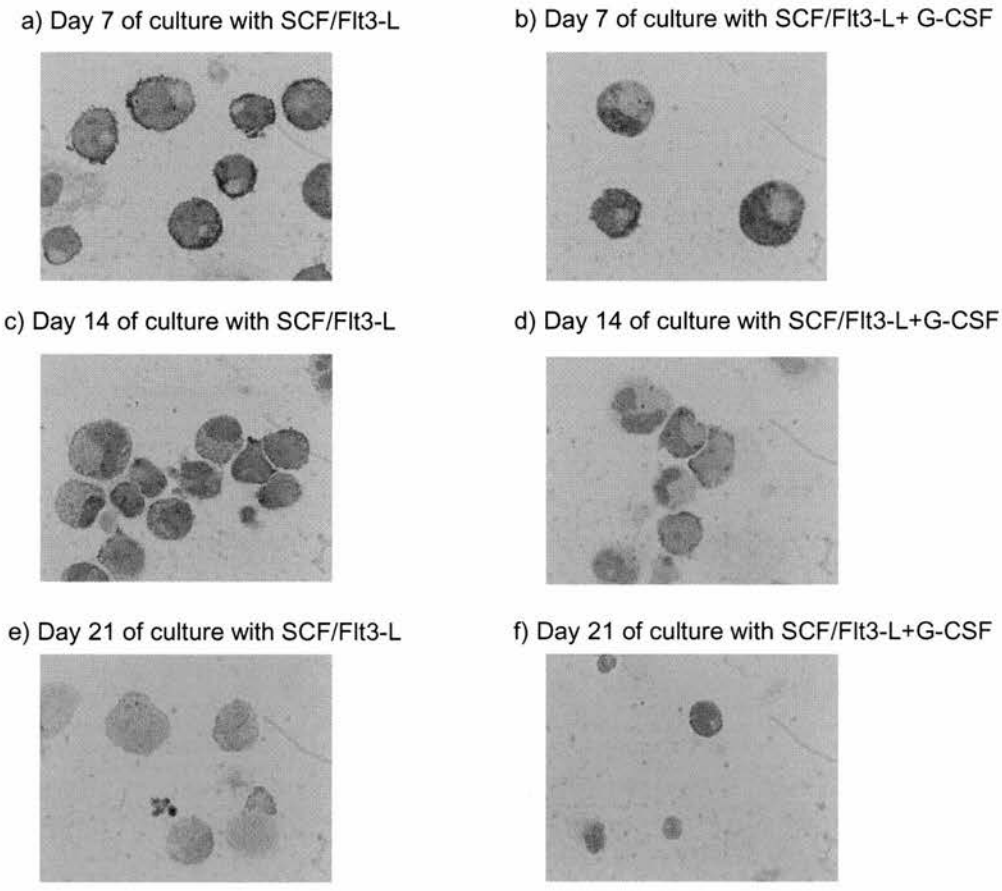


Figure 4.4 Morphology in CD34⁺ cells at 7 day intervals with/without G-CSF..

Cell morphology after 7, 14, and 21 days of culture with SCF/Flt-3L with or without G-CSF.

4.3.2 SHP-2 and PTEN expression

There is no or very weak expression of SHP-2 in peripheral blood neutrophils from healthy donors (I.Heinisch *et al*, unpublished data). In contrast to mature neutrophils, high levels of SHP-2 were detected in CD34⁺ expanded cells. There is a considerable reduction of the SHP-2 expression as the cells differentiate towards a more mature neutrophil cell population with a minimal level of expression at day 21. However, there was not a significant difference in expression between cells cultured in SCF/FLt3-L alone or with the addition of G-CSF or TPO (Figure 4.2). The expression of protein tyrosine phosphatase (PTEN) in CD34⁺ differentiated cells was comparable with that of mature neutrophils isolated from healthy adults (I.Heinisch *et al*, unpublished data) (data not shown).

4.3.3 Cord blood samples versus G-CSF mobilised samples

$CD34^+$ differentiated cells from cord blood or from G-CSF mobilised PBSC samples had the same SHP-1 and SHP-2 expression pattern (data not shown). All the samples used above were mobilised peripheral blood.

4.3.4 Transfection of $CD34^+$ differentiated cells

$CD34^+$ derived neutrophils were successfully transfected at different stages of maturation using the Amaxa electroporation system. Cells transfected with SHP-1-GFP construct resulted in increased induction of cell death in comparison with cells transfected with a control GFP construct (Figure 4.5).

The same result was seen using promyelocytic leukaemic HL-60 cells (data not shown).

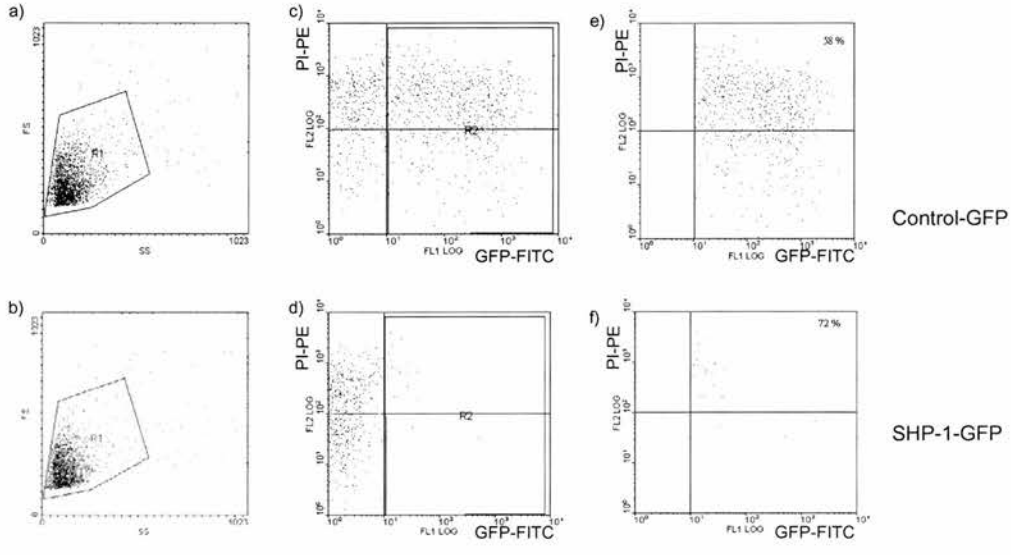


Figure 4.5 Transfection of $CD34^+$ differentiated cells

Ex vivo differentiated cells were successfully transfected. Flow cytometry analysis (a,b) FCS/SSC, (c,d) double staining with PI-PE and GFP-FITC, (e,f) R2 gated GFP-positive cells double staining with PI-PE and GFP-FITC. SHP-1 GFP expression induces cell death of these $CD34^+$-derived differentiated cells (72%) compared to 58% when the cells were transfected with control-GFP.

4.4 Discussion

Neutrophil turnover is very rapid and a tight homeostasis has to exist to guarantee control of functionality. An *ex vivo* protocol for the generation of neutrophils from $CD34^+$ cells was employed to simulate *in vivo* neutrophil differentiation. This was used to test if this tight equilibrium was due to the balance between the inhibitory phosphatase SHP-1 and the activating phosphatase SHP-2 and whether an unbalance equilibrium of these phosphatases is the cause of situations like leukaemia. Higher expression of SHP-2 over SHP-1 is detected in leukaemic cell lines whereas healthy mature neutrophils do not express SHP-2. *Ex vivo* generated neutrophil precursors at different stages of their differentiation process (at day 7 and 14) expressed SHP-2 with no apparent difference over the time period analysed. However, SHP-2 expression was hugely reduced at further culture times (day 21) when cells reached total maturity. SHP-2 is known to be an activating phosphatase and might be present while the cell is able to proliferate, possibly as a survival factor. At day 14 *ex vivo* expanded cells are still able to proliferate and SHP-2 expression might need completion of cell maturity to be switched off.

SHP-1 is expressed in healthy neutrophils at 70kDa. A different lower molecular weight form of SHP-1 (62kDa) can be found in both healthy individuals or in *ex vivo* generated neutrophil precursors. Myelocytic leukaemic cell lines only expressed the 70kDa SHP-1 band or no SHP-1 at all.

The two SHP-1 forms were detected in both *ex vivo* generated neutrophil precursors cultured with SCF/Flt-3L alone, with SCF/Flt-3L+G-CSF or with SCF/Flt-3L+TPO at day 7 and 14. We hypothesised that SHP-1, known as an inhibitory phosphatase (Tonks and Neel, 1996), would increase or maintain its expression during neutrophil maturation and together with a reduction of SHP-2 expression be responsible for inducing neutrophil cell death. Cells transfected with a SHP-1-GFP construct resulted in an increased induction of cell death. Leukaemic cell lines have a SHP-1/SHP-2 balance in favour of SHP-2 expression. Thus, we hypothesised that the loss of SHP-1 was the reason why leukaemic cell lines lost the ability to apoptose. However, SHP-1 expression did not increase during neutrophil maturation. SHP-1 expression was detected in very early stages of $CD34^+$ cell differentiation.

Two different SHP-1 bands were detected in our samples. Mature peripheral blood neutrophils expressed more of the lower molecular weight SHP-1 band (62kDa) than the higher molecular weight SHP-1 band (70kDa) whereas $CD34^+$ cells showed stronger expression of the 70kDa band compared to the 62kDa SHP-1 band. *Ex vivo* generated neutrophils showed a significant decrease of expression in the 70kDa

band as they differentiated towards the neutrophil lineage as well as a slightly decrease of the 62kDa band with almost no expression at day 21. Healthy neutrophils have a stronger expression of the 62kDa band over the 70kDa band. Therefore, expanded mature neutrophils at day 21 have a very distinct SHP-1 expression which is different from isolated healthy neutrophils. Further studies are needed to understand SHP-1 expression pattern though neutrophil differentiation. Whether a balance of the two SHP-1 forms regulates neutrophil cell death and what physiological consequences this might have is currently under investigation.

There are four different transcripts of this gene due to alternatively spliced mRNA. Therefore, the two bands could be due to differentially spliced mRNA transcripts. It is intriguing that leukaemic cell lines only expressed the 70kDa or no SHP-1 at all. Any relevance to leukaemia awaits further investigation.

In summary, the use of our *in vitro* $CD34^+$ neutrophil differentiation system is a good model to study changes in the expression of phosphatases that occur during neutrophil differentiation.

Chapter 5

Endothelial Progenitor Cells

5.1. Introduction

5.1.1. Study background

The recent work describing stem cells with regenerative capacity for a variety of tissues has encouraged a number of groups to pursue stem cell based solutions for clinical problems in specific tissues. However, in most cases it is not known if or where stem cells may reside in specific tissues, how to recognise such cells, how to access and harvest such cells for clinical procedures, and whether such cells persist in damaged or diseased tissue. Unlike cases where haematopoietic stem cell transplant can be employed, where there is innate or myeloablation-induced immunity deficiency which can permit allograft HSC use, in most other tissue repair circumstances the potential patients have intact immune systems which would reject allograft stem cells, restricting practical clinical solutions to autologous stem cells. Concurrent and compatible concepts that pluripotent stem cells originate in bone marrow and transit via circulating blood to sites where they are required for tissue repair, coupled with the success of bone marrow or blood sources of HSC in clinical haematopoiesis restoration and the availability of these sources for clinical use, has stimulated examination of bone marrow or blood mononuclear cells as sources of cells for repair of other tissues. While there is an accumulating body of experimental evidence for generation of a variety of tissue-differentiated cells from, especially, bone marrow, the precise cells from which such differentiated cells arise and the processes by which these outcomes take place remain obscure, as was discussed in the general Introduction (Chapter 1).

Many such differentiation phenomena have been described for *in vitro*

investigations, and have proved difficult to reproduce in experimental animal studies, let alone develop for clinical application. However, there are two emerging phenomena deriving from the bone marrow/blood repertoire which are beginning to build consistent reproducible characterisation in laboratory and animal experimentation and in clinical investigation and application. These are centred around mesenchymal stem cells (MSC) and on endothelial progenitor cells (EPC). The mesenchymal stem cells (or marrow stromal cells) are in part defined by their ability to differentiate into either bone, cartilage or fat under appropriate stimuli *in vitro*, and it is probable that their main clinical use will be in bone or cartilage regeneration. However, they have been attributed with the ability to differentiate into a variety of other tissues, including endothelium, and therefore they will be discussed tangentially below (see 5.1.10). The endothelial progenitor cells are the focus of the remainder of this thesis, and will be reviewed in detail.

Current concepts of endothelial progenitor cells and their role in vascular repair and regeneration represent a convergence of perspectives from transfusion medicine, transplant immunology and haematology, including HSC transplantation, which is the background of our laboratory and clinical interests, with perspectives from cardiovascular experimental and clinical research and from recent embryology and developmental biology developments. These have caused a paradigm shift from the concept that in post natal life vascular repair and regeneration arose only from outgrowth and budding from existing mature vasculature, to a recognition that significant vascularisation and angiogenesis results from a contribution from endothelial progenitor cells (EPC) requisitioned from a circulating population that may originate in bone marrow from a common precursor of EPC and HSC.

It has been known for many years that during embryo development blood and the vascular system develop from a common stem cell termed the haemangioblast. The recent demonstration by Asahara *et al.*, (1997) that a subpopulation of cells apparently within the HSC ($CD34^+$) population can differentiate into endothelial cells (EC) has stimulated a range of experimental studies which support the existence and role of endothelial progenitor cells and their precursor, a haemangioblast with dual haematopoietic and vasculogenic potential, in the adult. This has also stimulated clinical trials employing HSC sources to remedy tissue ischaemia, which have on the whole indicated clinical benefit. In some cases such as critical limb ischaemia increased vascularisation can be demonstrated directly. In other cases such as myocardial ischaemia the recovery of myocardium might be due to a contribution by myocardial stem cells or transdifferentiating HSC in the locally injected autograft, as was originally sought, but is increasingly being attributed to

revascularisation by the autograft of infracted tissue permitting myocardial recovery from dormant local myocardial cells. This is supported by post-mortem or biopsy examination of tissue from solid organ or HSC allograft recipients, where the most extensive circulatory contribution of crossover cells to local tissue is to vascular tissue where e.g. in transplanted heart host cells may reach almost 25% of vascular endothelial cells (Minami *et al.*, 2005).

While increasing numbers of clinical trials indicate benefit in both cardiac and critical limb ischaemia, such trials have mostly employed autologous haematopoietic stem cell sources, mainly bone-marrow, but also G-CSF mobilised PBSC. While such sources can be assessed for haematopoietic potential by $CD34^+$ cell content, there is no equivalent agreed phenotype definition for EPC or assessment criteria for clinical efficacy. Intriguingly there are reports in which $CD34^+$-low cultured normal peripheral blood MNC (not HSC enriched by G-CSF infusion) are as efficacious as $CD34^+$-high fresh bone-marrow MNC in autograft treatment of acute myocardial infarct cardiac by intracoronary infusion (Assmus *et al.*, 2002). Neither is there any consensus on which cell, or cells, in the graft are critical for mediating vascular repair, nor which source (bone-marrow, normal peripheral blood MNC, mobilised PBSC) is best, which agent may mobilise EPC to the circulation, whether subpopulation-enriched cell sources are better clinically, or whether clinical performance can be enhanced by *ex vivo* stimulation.

In the face of an uncertain phenotype, the EPC colony forming unit assay (CFU-EPC) has emerged as an alternative specific enumeration system for EPC. Hill *et al.*, (2003) first described a version of this colony assay based on spontaneous mononuclear cell colony outgrowth on fibronectin coated plates in simple medium without growth factors, in which these colonies stained for mature endothelial markers. Hill *et al.*, (2003) used the assay to assess whether the level of circulating endothelial progenitor cells correlated with the presence or absence of conventional cardiovascular risk factors (Hill *et al.*, 2003). This colony assay is available as a standardized commercial kit which should allow inter-laboratory comparison of results, and has been applied in a number of recent studies of different clinical conditions either alone (Hill *et al.*, 2003; Burnham *et al.*, 2005; Ghani *et al.*, 2005; Hoetzer *et al.*, 2005; Sugawara *et al.*, 2005) or together with phenotype studies (Grisar *et al.*, 2005; Laufs *et al.*, 2005; Lev *et al.*, 2005), to demonstrate increased or reduced numbers of circulating endothelial progenitor cells.

In this thesis we compare available putative autologous EPC sources by phenotype and *in vitro* functional characteristics which may indicate parameters for prediction of clinical efficacy of these sources, and also in Chapter 6 study expression of these

characteristics in certain clinical groups who may be candidates for EPC autograft therapy.

5.1.2.The haemangioblast as precursor of both HSC and EPC

5.1.2.1 The embryonic haemangioblast

Vasculogenesis is *de novo* formation of blood vessels from the *in situ* differentiation of undifferentiated mesodermal cells into endothelial cells. Mesodermal precursor cells named haemangioblasts were first identified in chick blastoderms (Sabin, 1920; Murray, 1932). In culture these cells were shown to produce both blood and endothelial cells (Murray, 1932). It was proposed that endothelial and haematopoietic stem cells due their close development within the yolk sac arise from a common precursor; the hemangioblast progenitor cell (Risau and Flamme, 1995; Tavian *et al.*, 1996; Bailey and Fleming, 2003; Oberlin *et al.*, 2002; Asahara *et al.*, 1997). Classical studies in developmental biology have used amphibians to examine embryogenesis but the general principles of embryonic development are maintained in higher organisms (Zon, 1995). The process begins in the embryo with the formation of cell clusters or blood islands, which initially contain a homogenous collection of progenitor cells, presumed to be haemangioblasts. With development, endothelial progenitor cells (EPCs) are located at the periphery of the blood islands and an inner layer of red blood cell precursors haematopoietic stem cells (HSCs) are found in the centre of the blood islands (Cogle and Scott, 2004). The process of haematopoiesis begins in the yolk sac blood islands, which primarily producing nucleated red blood cells and goes in the intra-embryonic sites of blood produce (Zon, 1995; Yoder and Hiatt, 1997; Kennedy *et al.*, 1997). Concurrently the angioblasts derived from endothelial progenitors begin to form lumens via a process called vasculogenesis. Later in development, an area described as the para-aortic splanchnopleura mesoderm contains haemangioblasts, giving rise to both intra-embryonic blood vessels and definitive haematopoiesis (Dieterlen-Lievre *et al.*, 2002). Growth and fusion of the multiple blood islands ultimately give rise to the capillary network structure (Cogle and Scott, 2004). After that, this network differentiates into an arteriovenous vascular system; EPCs give rise to vascular ECs, whereas HSCs develop into mature blood cells (Cogle and Scott, 2004).

Investigation of development of the early human embryonic haematopoietic and vascular systems showed similar results. The accumulation of $CD34^+$ cells densely clustered on the ventral wall of the developing aorta was seen during the fifth week of gestation (Tavian *et al.*, 1996). Phenotypic characterization of these intra-aortic

clusters revealed both haematopoietic and endothelial cells. Moreover highly purified human embryonic endothelial cells were shown to produce both myeloid and lymphoid haematopoietic colonies (Oberlin *et al.*, 2002).

The differentiation of HSC and EPC appear to be closely related both temporally and physically. Both share a number of surface markers in the developing yolk sac and embryo and genetic disruption of numerous genes affects haematopoietic and endothelial development (Ingram *et al.*, 2005). Targeted gene mutation studies using the ES *in vitro* culture system have been used to evaluate the molecular signals that specify haematopoietic and endothelial outcomes. This approach has identified several genes such as Flk-1, cloche, SCL, Runx1 which may play important roles in the development of both endothelial and haematopoietic systems (Bailey and Fleming, 2003).

Flk-1 deficient mice embryos die in utero between days 8.5 and 9.5 as a result of haematopoietic and endothelial defects (Shalaby *et al.*, 1995). Mutation of the cloche gene in zebrafish affects both endothelial and haematopoietic lineages at a very early stage (Stainier *et al.*, 1995). Notably these mutant embryos are both deficient in viable blood cells and lack an endothelial lining of the heart (Cogle and Scott, 2004). SCL-/- embryos contained no primitive or definitive HSCs in the yolk sac although they develop a primary vascular network (Robb *et al.*, 1995; Shivdasani *et al.*, 1995). On the other hand, Runx-1 deficient embryos (a mouse homologue to human AML-1) produce normal blood islands and develop primitive erythrocytes but they have a complete block in definitive haematopoiesis resulting in death between day 11 and 13 of gestation (Wang *et al.*, 1996; Okuda *et al.*, 1996).

HSCs appear to be critical for early embryonic blood vessel development. Wild-type HSCs rescue impaired angiogenesis in acute myeloid leukaemia 1 (AML-1)-deficient embryos (Takakura *et al.*, 2000).

Several lines of investigation demonstrated that many of the genes activated during early haematopoietic development are also expressed in the vascular endothelium such as Flk-1, Tie-2, c-Kit, Sca-1, CD133 and CD34 (Murasawa and Asahara, 2005). *In vitro* ES cells express genes common to both haematopoietic and endothelial cell lineages including Flk-1 and CD34. ES cell derived embryoid bodies (EB) give rise to a transient precursor blast colony forming cell (BL-CFC) which when differentiated can generate primitive erythrocytes, multilineage haematopoietic cells and an adherent endothelial population (Kennedy *et al.*, 1997 Choi, 2002). However, due to the low frequency of BL-CFC it is yet to be determined if all these subpopulations are produced by a single EB-derived cell (Cogle and Scott, 2004).

5.1.2.2 Postnatal vascularisation

Vasculogenesis also contributes to postnatal vascularisation, which requires the mobilisation of bone marrow-derived endothelial stem cells, which home to sites of ischaemia, and contribute to new blood vessel formation. Until recently, it was thought that blood vessel formation in postnatal life was only mediated by sprouting of endothelial cells from existing mature vessels (angiogenesis) (Reyes *et al.*, 2002). The finding that vasculogenesis also exists in the adult life offers novel therapeutic strategies for the use of circulating endothelial progenitor cells or their precursors for cell therapy of tissue-ischaemia (Urbich and Dimmeler, 2005).

Adult blood vessel formation also occurs through other processes such as arteriogenesis or angiogenesis. Arteriogenesis describes the growth of collateral vessels, whereas angiogenesis refers to the growth of new capillaries to carry blood to ischaemic tissues by sprouting of pre-existing vessels through migration and proliferation of mature endothelial cells under the control of multiple factors in the embryo and in the adult life (Urbich and Dimmeler, 2005; Moldovan *et al.*, 2000).

5.1.2.3. Evidence for an adult hemangioblast

In contrast to embryonic development, the complex functional relationships between haematopoiesis and the vascular compartment in postnatal life have only recently been investigated (Montfort *et al.*, 2002). For many years, it had been assumed that the haemangioblast was a transient bipotent cell restricted to the developing blood and vascular system of the yolk sac and in the early embryo (Bailey and Fleming, 2003). Recent evidence suggests that a sub-set of progenitor cells having a function resembling that of the embryonic haemangioblast is present in adult bone marrow. These cells, and their endothelial lineage progeny termed endothelial progenitor cells (EPC) can be actively recruited to the peripheral circulation, can migrate to ischaemic tissues and to tumour microenvironments and can differentiate into mature endothelial cells (Ingram *et al.*, 2005; Zammaretti and Zisch, 2005; Asahara *et al.*, 1997; Asahara et al. (a),1999; Hristov *et al. (a)*, 2003).

The first evidence for an adult stem cell contributing to blood vessel production was reported in 1997 when Asahara *et al.* identified in humans circulating EPCs contributing to active sites of neovascularisation. Subsequently Raffi's group (1998) reported the existence of circulating bone marrow derived endothelial progenitor cells in the adult (Shi *et al. (a)*, 1998). Most convincingly, evidence for a bone marrow derived circulating adult EPC has come from transplantation of specific subsets of bone marrow cells (Shi *et al. (a)*, 1998; Shi *et al. (b)*, 1998). Shi *et al.*, (1998) demonstrated that a subset of transplanted human bone marrow derived cells

participated in the endothelisation of implanted Dacron grafts in a canine model of vascular graft healing (Shi *et al. (a)*, 1998; Shi *et al. (b)*, 1998). Transplantation of adult mouse bone marrow side population cells (SP) into irradiated mice, followed by myocardial injury, resulted in donor-derived endothelial cells in the region of injury repair (Jackson *et al.*, 2001). Moreover Grant *et al.*, (2002) using serial long-term transplants, in a mouse injury model of diabetic retinopathy, to ensure that activity arose from self-renewing stem cells, found that clonal progeny of a single bone marrow (c-Kit$^+$, Sca-1$^+$, lineage-negative) cell (KSL) exhibited functional haemangioblast activity. These clonally derived cells differentiated into all haematopoietic cell lineages as well as endothelial cells with the formation of donor-derived functional blood vessel which integrated into the vascular system. Moreover, mice transplanted with bone marrow derived KSL cells following irradiation were found to have donor derived endothelial cells in many tissues (Bailey *et al.*, 2004). Cell fusion was excluded as the principal mechanism responsible for the generation of functional endothelial cells in these two cases.

Transplanted EPCs incorporated into sites of active angiogenesis and differentiated into mature ECs with clear benefits of augmentation of angiogenesis and collateralisation in animal models of tissue ischaemia (Kalka *et al. (a)*, 2000; Kawamoto *et al.*, 2001; Murohara *et al.*, 2000).

Furthermore, Reyes *et al.*, (2002) have isolated a population of multipotent adult progenitor cells (MAPC) from human bone marrow, which in culture differentiated into cells with phenotypic and functional characteristics indistinguishable from mature endothelial cells. *In vitro* generated MAPC-derived endothelial cells responded to angiogenic stimuli by migrating to tumour sites and contributed to tumour vascularisation in a mouse model of tumour and wound neoangiogenesis (Reyes *et al.*, 2002).

Some human HSC subsets such as CD34$^+$VEGFR2$^+$ cells have been shown to generate, at a single cell level, both haematopoietic and endothelial cells in culture (Pelosi *et al.*, 2002).

All these findings support the hypothesis that cells with hemangioblast activity reside within adult bone marrow. However, in the absence of a definitive haemangioblast phenotype it is impossible to be certain that *de novo* vascularisation following transplantation of specific subsets of bone marrow cells is not due to HSC or EPC, as all these cell types share expression of surface markers. To address the question of haemangioblast activity in the adult, single cell transplantation experiments must be performed in order to demonstrate the existence of a cell that is capable of generation of both haematopoietic and endothelial cell outcomes (Bailey and Fleming,

2003).

It will also be important to evaluate the interaction between blood vessels and blood production. There is preliminary evidence that HSC transplantation efficiency increases when HSC are co-cultured with endothelial cells, (Chute *et al.* (a), 2004; Chute *et al.* (b), 2004). These studies indicate that blood vessels either prime HSC by providing survival and proliferation factors or directly serve as a haematopoietic repopulation cell source. However it is yet to be determined if the vascular endothelium has the potential to differentiate into haematopoietic cells (Cogle and Scott, 2004).

Currently, it is thought that EPCs involved in neovascularisation are bone marrow-derived cells which are mobilised into the systemic circulation in response to certain cytokines and/or tissue ischaemia and home into sites of neovascularisation. However, it is possible that resident tissue-specific stem cells are responsible for regenerating damaged tissue and maintaining tissue homeostasis. Recently tissue resident c-Kit$^+$ cells isolated from the heart have been shown to differentiate into endothelial lineage cells (Beltrami *et al.*, 2003).

Similarly, Wurmser *et al.*, (2004) demonstrated that neural stem cells could differentiate into endothelial lineage cells *in vitro* and *in vivo*. These findings suggest that it is possible that stem cells remain in some/all tissues and organs beyond embryonic development and that these stem cells can self-renew and generate cells of different lineages in case of cell damage and senescence (Guo *et al.*, 2003).

5.1.3 Identification of endothelial progenitor cells

Interest in circulating angiogenic cells comes from at least 30 years ago when circulating cells with endothelial cell morphology were isolated from a variety of experimental models of vascular injury (Wright and Glacometti, 1972; Yarnal and Hollis, 1974). Further investigation was hampered by the lack of reagents to specifically identify these cells as endothelial cells. Even though definitive marker(s) for endothelial cells have still to be identified the discovery of monoclonal antibodies and the use of magnetic bead-immunoselection and/or fluorescence-activated cell sorting have improved our ability to isolate, enumerate and characterize endothelial progenitor cells (EPCs) and circulating mature endothelial cells (CECs). Even though, these cells remain very rare in the adult peripheral blood (0.01% CECs and 0.0001% EPCs) (Ingram *et al.*, 2005).

Typically, mononuclear cells are isolated from bone marrow, peripheral blood and cord blood by density gradient centrifugation. These may then be subpopulation-enriched by separation by magnetic beads or by flow cytometry with antibodies

against surface antigens such as CD34 or CD133. Although there has been an intense effort to define EPC and CEC cell populations, there is still no consensus on their exact phenotype definition and it is still unclear what cell type(s) induced angiogenesis (Ingram *et al.*, 2005). Given the similarities between blood and blood vessel production in the embryo, HSCs were initially proposed as candidates for an EPC precursor in the adult (Asahara *et al.*, 1997). The EPCs identified by Asahara *et al.*, 1997 were in a fraction of adult bone marrow which comprised 15% $CD34^+$ cells. *In vitro* these cells generated spindle-shaped cells, defined as endothelial cells based on morphology, expression of surface markers and immunohistochemical staining. The circulating EPC reported by Shi *et al.*, (1998) were also derived from a $CD34^+$ HSC subset. These cells differentiated to the endothelial lineage, expressing von Willebrand factor (vWf) and incorporated acetylated low-density lipoprotein (ac-LDL). Similarly, the endothelisation of Dacron grafts implanted in a canine model of vascular injury was derived from a $CD34^+$ HSCs bone marrow subset (Shi *et al. (a)*, 1998; Shi *et al. (b)*, 1998). According to these initial discoveries, EPCs were defined as cells that share HSC markers such as CD34 and also express a more endothelial specific marker such as vascular endothelial growth factor receptor 2 antigen (VEGFR2) also termed (in humans) kinase insert domain receptor (KDR) (equivalent to Flk-1 in mice).

Since then, attempts to define and quantify the number of EPCs in different experimental and clinical situations have grown year by year. However, it is still difficult to distinguish an immature EPC from primitive HSCs, due to their shared expression of common markers. Furthermore, neither of these markers are specific for EPCs alone or together because VEGFR2 and CD34 are also expressed by mature endothelial cells. The lack of specific EPC markers complicates the detection and quantification of true EPCs in the circulation. The discovery of CD133 permits the detection of more primitive HSCs (www.miltenyibiotec.com). During maturation CD133 expression is lost by both EPCs and HSCs, whilst VEGFR2 expression is maintained only by EPCs throughout maturation (Hristov *et al.* (b), 2003). Therefore, hypothetically, the co-expression of these three markers ($CD34^+CD133^+VEGFR2^+$) could identify an EPC population whereas positivity for CD34 and CD133 but not for VEGFR2 (i.e. $CD34^+CD133^+VEGFR2^-$) could be a more appropriate definition of an HSC (Hristov *et al.* (b), 2003). Through differentiation EPCs begin to express more mature endothelial markers such as VE-cadherin, CD31 (PECAM), CD146 (also known as S-endo), endothelial NO synthase, E-selectin and Von Willebrand factor (Hristov *et al.* (b), 2003). Moreover, mature endothelial cells are supposed to be CD45 negative (CD45 is expressed in all haematopoietic cells), actively take up

the low-density lipoprotein (acetylated LDL) and bind ulex-lectin (UEA-1).
Currently, every group uses their own EPC definition, combining one, two or three of these antigens (CD34/CD133/VEGFR2); summarised in Table 5.1. It is established that the EPC population is in the mononuclear fraction of the total whole blood cells, and accepted that EPCs share the CD34 marker with haematopoietic stem cells. Some groups use expression of CD34 as their definition of an EPC, without regard to co-expression of other markers (Asahara *et al.*, 1997; Murohara, 2001; Shi *et al.* (a), 1998). Others prefer the isolation of $CD133^+$ cells as a better approach since it discards any mature CEC which retain CD34 expression. For example some $CD133^+$ isolated cells differentiate into ECs *in vitro* under appropriate culture conditions (Gehling *et al.*, 2000; Kocher *et al.*, 2001). Gehling *et al.*, 2000 showed that enriched $CD133^+$ cells from G-CSF mobilised peripheral blood can be differentiated along both endothelial and haematopoietic pathways. They also demonstrated that injection of $CD133^+$-derived cells together with tumour cells enhanced tumour growth though tumour vascularisation and the vascular component was derived from the injected human $CD133^+$ cells. Overall, isolation of $CD133^+$ stem cells provides a selective population useful for transplantation and tissue regeneration (Shmelkov *et al.*, 2005). Most importantly, injection of $CD34^+$ or $CD133^+$ cells enhanced neovascularisation in animal models following ischaemic injury (Asahara and Kawamoto, 2004; Hofmann *et al.*, 2005) and recently, transplanted $CD133^+$ bone marrow cells have been shown to improve function of infarcted myocardium in human patients (Stamm *et al.*, 2003). However, whether enriched $CD34^+$ and/or $CD133^+$ cells consist of a mixture of separate progenitors for endothelial and haematopoietic cells, or whether there is a common precursor for both lineages remains unknown.
Other groups have defined EPCs as a subset of $CD34^+$ cells also expressing VEGFR2 (KDR) antigen (Botta *et al.*, 2004; Pelosi *et al.*, 2002). Pelosi *et al.*, (2002) identified a "postnatal haemangioblast" in a $CD34^+KDR^+$ cell subset with long term proliferative potential and bi-lineage differentiation capacity. Alternatively, some studies have recently demonstrated that $CD133^+VEGFR2^+$ cells in the circulation have the capacity to migrate and differentiate into adherent mature endothelial cells (Gehling *et al.*, 2000; Peichev *et al.*, 2000; Quirici *et al.*, 2001). Whereas others prefer to use a triple positive EPC definition ($CD34^+CD133^+VEGFR2^+$) (Peichev *et al.*, 2000; Vasa *et al.*, 2001). Peichev *et al.*, (2000) demonstrated that $CD34^+$ cells co-expressing CD133 and VEGFR2 have the capacity to migrate and differentiate into adherent mature endothelial cells. It is very difficult to interpret and compare results between such studies because each group uses their particular EPC definition based on their

experimental and/or clinical outcomes.

Author	source	cell source	CD31	vWf	VEGFR2	ac-LDL	Ve-cad	UEA-1	CD34	e-Nos	CD45	CD133	CD14	CD105	Tie-2	CD146	Matrigel	clinical + outcome	Propose EPC definition
Boyer *et al*, 2000	CB/PB	CD34+	+	+	+				+										CD34+
Murohara *et al*, 2001	CB	CD34+	+		+	+	+			+	X								CD34+
Fan Chun-ling *et al*, 2003	CB	CD34+	+	+		+													CD133+VEGFR2+
Pelosi *et al*, 2002	CB	CD34+VEGFR2+	+			+	+				X		X				+		CD34+VEGFR2+
Botta *et al*, 2004	CB	CD34+VEGFR2+																+	CD34+VEGFR2+
Murohara *et al*, 2001	CB	MNCs	+	+		+				+									MNCs
Asahara *et al*, 1997	PB	CD34+(15%purity)	+		+	+									+				CD34+
Fernandez-Pujol *et al*, 200	PB	CD14+		+	+	+	+							+			+		CD14+
Urbich *et al*, 2003	PB	CD14+/-		+	+	+		+		+	+			+			+	+	cultured CD14+/-
Harraz *et al*, 2001	PB	CD34- and CD14+CD34-			+		+			+	+				+				CD34-/CD14+
Scatteman *et al*, 2000	PB	CD34+																+	CD34+
Schmeisser *et al*, 2001	PB	CD34-CD14+		+			+			+	+						+		CD34-CD14+
Lin *et al*, 2000	PB	MNCs	+	+	+	+	+		+				+			+			MNCs
Kalka *et al*, 2000	PB	MNCs	+		+	+	+	+	+				+						MNCs
Gill *et al*, 2000	PB	MNCs			+		+					+							CD133+VEGFR2+
Vasa *et al*, 2001	PB	MNCs		+	+	+	+	+											CD34+VEGFR2+ or CD34+CD133+VEGFR2+
Dimmeler *et al*, 2001	PB	MNCs	+	+	+	+	+	+		+									
Kawamoto *et al*, 2001	PB	MNCs				+												+	MNCs
Rehman *et al*, 2004	PB	MNCs				+		+											CD133+VE-CAD+
Gulati *et al*, 2003	PB	MNCs (rabbit)	+			+				+			+					+	MNCs (monocytic?)
Kaushal *et al*, 2001	PB	MNCs (sheep)	+	+	+	+									+		+	+	MNCs
Takahashi *et al*, 1999	PB	Sca-1+ (mice)	+														+		Sca-1+
Gehling *et al*, 2000	MB	CD133+		+	+			+					X		+			+	CD133+
Quirici *et al*, 2001	BM	CD133+		+		+	+	+	weak	+	some	X	some			+			CD133+
Quirici *et al*, 2001	BM	CD133+ and UEA-1+		+			+				X		X	+		+			CD133+/CD34+VEGFR2+
Shi *et al*, 1998	BM	CD34+		+		+												+	
Grant *et al*, 2002	BM	HSC(c-Kit+,Sca-1+,Lin-)																+	c-Kit+,Sca-1+,Lin-
Bailey *et al*, 2004	BM	HSC(c-Kit+,Sca-1+,Lin-)	+	+		+												+	HSC
Asahara *et al*, 1999	BM	MNCs																+	MNCs
Tateishi-Yuyama *et al*, 20(	BM	MNCs																+	MNCs
Guo *et al*, 2003	BM (fetal)	VEGFR2+CD31-CD34-	+	+	+														
																			HSC
Reyes and Verfaillie, 2001	BM	MAPC		+	+	+	+		+								+		MAPC
Jiang *et al*, 2002	BM/Brain/muscle	MAPC	+	+															MAPC
Peichev *et al*, 2000	FL	CD34+				+													CD34+VEGFR2+CD133+
Planat-Benard *et al*, 2004	Adipose tissue	Adipocyte cells																+	Adipocyte cells
Jackson *et al*, 2001	BM	SP (CD34-,C-Kit+,Sca-1+)																	SP cells

Table 5.1 Summary table of different proposed EPC definitions

Each group uses their own EPC definition, combining one, two or three of these antigens (CD34/CD133/VEGFR2).

+ positive; X negative; blank not tested.

5.1.4 Sources of endothelial progenitor cells

Identification of suitable EPC sources is of the highest importance as a prerequisite for any clinical EPC use. If HSCs are the source of EPCs in adult life, sources rich for HSCs are going to be the best candidates as an EPC reservoir. Bone marrow, G-CSF mobilised blood and cord blood are sources with the highest number of HSCs cells. In an autologous clinical context freshly isolated bone marrow mononuclear cells, HSC-mobilised peripheral blood mononuclear cells, and the $CD34^+$-enriched or $CD133^+$-enriched subpopulations of these have been injected locally in infarcted myocardium or ischaemic limbs with, in most cases, some recorded clinical benefit (see table 5.1.17 and table 5.2). While it cannot be used for autologous EPC transplant, cord blood has also high numbers of HSCs and transfusion of severe combined immunodeficiency (SCID) mice with human cord blood has demonstrated repopulation of the bone marrow (Murohara, 2001). Cord blood HSCs were differentiated to ECs in several experiments (Peichev *et al.*, 2000; Murohara *et al.*, 2000). Pesce *et al.*, (2003), showed that $CD34^+$ cord blood cells injected into mouse ischaemic adductor muscles gave rise to endothelial and to skeletal muscle cells. Also, Boyer *et al.*, (2000) showed that endothelial progenitor cells can be isolated from cord blood and used to generate EC cultures as a source of cells for vascular graft seeding and gene therapy. Currently, cord blood transplantation is allogeneic, therefore CB-derived EPC could induce immunological reactions and thus implanted cells would be rejected, in the absence of immunosuppression by the host's immune defence mechanisms.

Shi *et al.* (a), (1998) proposed G-CSF administration as a therapy to mobilize and increase EPCs in circulation since this is commonly used for HSC mobilisation. Infusion of peripheral blood stem cells obtained by aphaeresis after G-CSF administration was shown to increase vascularisation in patients after myocardial infarction (Kang *et al.*, 2004) and in limb ischaemia (Kawamura *et al.*, 2005). Suzuki *et al.*, (2003) showed that G-CSF growth factor manipulation would be a low invasive harvesting method and a good therapeutic strategy to ensure sufficient amounts of progenitor cells for clinical application.

Peripheral blood, which is not rich in HSC, has been suggested as a possible source of endothelial progenitor cells (Assmus *et al.*, 2002). Peripheral blood MNCs were enhanced *ex vivo* by culture on fibronectin and with the addition of growth factors before use. In a comparative clinical trial of peripheral blood derived EPC versus bone marrow derived EPC, implantation of both cell preparations into infarcted myocardium gave similar positive effects showing an improvement of regional myocardial contractility and viability (Schachinger *et al.*, 2004). Peripheral blood

has been generally overlooked as a source or stem cells because its low number of HSCs. However it may have better potential than previously expected (Assmus *et al.*, 2002).

5.1.5 Gene expression profiling from various haematopoietic stem cells sources

Comparative analysis of gene-expression profiles showed differential expression of genes between different HSC sources. Several studies have demonstrated that cord blood $CD34^+$ cells and G-CSF mobilised peripheral blood $CD34^+$ cells significally differ from bone marrow $CD34^+$ cells qualitatively and quantitatively (Ng *et al.*, 2004). Ng *et al.*, (2004) showed that 51 genes differ between bone marrow and cord blood stem cells and 64 genes between bone marrow and G-CSF mobilised peripheral blood. The differential genes were mainly transcription factors, involved in cell-cycle (proliferation, differentiation and apoptosis) and genes involved in stem cell homing and adhesion. Few, if any, $CD34^+$ G-CSF mobilised cells are in S phase, in contrast to 30% to 60% of bone marrow $CD34^+$ cells. The low expression of classic markers of S phase and G2-M transition detected in G-CSF mobilised $CD34^+$ cells in array analysis are in agreement with this quiescent phenotype (Graf *et al.*, 2001). Cord blood $CD34^+$ cells and G-CSF PB CD34+ cells showed reduced expression of elastase, myeloperoxidase, and cathepsin G and lower CXCR4 than bone marrow $CD34^+$ cells. G-CSF mobilised $CD34^+$ cells also display a decrease of expression of many cytokines, such as IL-8 (Graf *et al.*, 2001).

5.1.6 Myeloid cells as EPC precursors.

Evidence is accumulating which demonstrates that endothelial cells can be generated from bone marrow derived myeloid cells (Elsheikh *et al.*, 2005; Schmeisser *et al.*, 2001; Urbich *et al.*, 2003; Gulati *et al.*, 2003; Rehman *et al.*, 2003; Harraz *et al.*, 2001). Harraz *et al.*, (2001) showed that CD34-negative $CD14^+$ monocytic cells had the capacity to differentiate into endothelial cells. Moreover, Schmeisser *et al.*, (2001) showed that only $CD14^+CD34^-$ cell subfractions co-express endothelial and monocyte/macrophagocytic markers and form tube-like structures *ex vivo*. Likewise, infusion of bone marrow derived $CD14^+CD34^-$ cells contributed to endothelial regeneration, which was functionally active as shown by release of nitric oxide (NO) (Fujiyama *et al.*, 2003). Additionally, *ex vivo* expansion of purified $CD14^+$ mononuclear cells yielded cells with endothelial characteristics, which *in vivo* incorporated in newly formed blood vessels (Urbich *et al.*, 2003), and Fernandez-Pujol *et al.* (2000) showed that under the appropriate culture conditions $CD14^+CD34^-$

cells differentiate into EC-like cells exhibiting characteristics of both endothelial cells and monocytes. Further, Ingram *et al.*, (2005) suggested that monocyte-derived EPCs have a similar capacity for augmenting neovascularisation as do HSCs or cord blood derived EPCs in experimental models.

However, monocytes were thought to be mature stage cells with a limited potential to proliferate in culture. Recently, it has been recognised that monocytes and macrophages are difficult cell types to define in end stage terms. In fact, these cells seem to be extremely flexible in their phenotype and function and could be continuously adapting in response to changing microenvironmental states. Rohde *et al.*, (2005) demonstrated that recognised endothelial markers such as ac-LDL uptake, lectin binding, and CD31, CD105, CD144 positive expression are also genuine features of blood monocytes. Indeed, primary monocytes already expressed most tested endothelial genes and proteins. Thus, this could indicate a closer relationship and a possible common origin than previously thought between endothelial and monocyte-macrophage lineage cells (Harraz *et al.*, 2001).

Monocytes/macrophages are in intimate contact with endothelial cells of the blood vessels and it is generally accepted that monocyte/macrophages can influence angiogenesis by secretion of growth factors. However, it seems that they can also have a more direct role in endothelial cell differentiation inducing neovascularisation of injured vessels and in tumour vasculogenesis (Fernandez-Pujol *et al.*, 2000). If monocytes/macrophages are involved in the process of capillarisation not only by the secretion of angiogenic growth factors but also by converting into a cell type at least similar to endothelial phenotype, with the potential to form vascular channels, this may offer new possibilities for diagnostic and therapeutic management of patients with ischaemic diseases (Schmeisser *et al.*, 2001).

5.1.6.1 CD14

CD14 was first described as a myeloid differentiation antigen in 1981 (Hailman *et al.*, 1994). It is a 55-KDa glycoprotein with multiple leucine-rich repeats and is encoded on chromosome 5 (5q) together with granulocyte colony stimulating factor. CD14 is an important component of the innate immune system and has been identified as the receptor for Gram-negative bacterial endotoxin (or lipopolysaccharide, LPS). It is also implicated in initiating septic shock, through interaction of CD14 with LPS complexed with LPS-binding protein (LBP) from plasma, but it also binds to other bacterial products (Hailman *et al.*, 1994, Kusunoki *et al.*, 1995). Interestingly, while endothelial cells are sensitive to low concentrations of LPS, it has been generally accepted that endothelial cells do not express CD14 (Beekhuizen, *et al.*, 1991).

However, Jersmann *et al.*, 2001 showed that vascular endothelial cells synthesized and expressed CD14 both *in vitro* and *in vivo* on the cell surface and presented evidence that the endothelial membrane-bound CD14 was functional in LPS-mediated cell activation. However compared to monocytes, the number of CD14 molecules expressed on HUVEC was small.

5.1.7 Mature endothelial cells.

Endothelial cells (ECs) are a crucial component of the normal vascular wall, not only providing a dynamic interface between blood and the extravascular matrix of the blood vessel wall, but also as a source of molecules that influence both the structural and functional integrity of vessel wall permeability and circulation (Muller *et al.*, 2002). ECs are intimately involved with the processes of angiogenesis, inflammation and thrombosis and there exists a considerably heterogeneity both structurally and functionally of the vascular monolayers between different organs (Cines *et al.*, 1998).

ECs secrete a wide variety of molecules involved in formation of platelet and fibrin thrombi such as von Willebrand factor (vWf). Indeed this monolayer of endothelial cells acts as a non-adherent surface for platelets and leucocytes, is involved in immune reactions and produces a variety of important regulatory factors such as prostaglandins and nitric oxide (NO) (Cines *et al.*, 1998). Von Willebrand factor, a multimeric glycoprotein synthesized exclusively in ECs and megakaryocytes and stored in Weibel-Palade bodies, is released from ECs and mediates initial platelet adherence to the subendothelium by linking to specific platelet membrane receptors (Mannucci, 1995; Muller *et al.*, 2002). Mature endothelial cells also express PECAM-1 (CD31), a 130 kDa transmembrane glycoprotein that plays a major role in a number of cellular interactions. CD34 antigen is also express on mature endothelial cells however, little is known about the significance and modulation of its expression (Muller *et al.*, 2002).

Unbalanced angiogenesis, resulting from excess or deficiency of blood vessel formation is associated with certain disease states. Diseases such cancer, diabetic retinopathy and rheumatoid arthritis are characterised by excessive blood vessel formation. Peripheral and coronary ischaemia and infarction, chronic wound healing failure and diabetic ulcers are characterized by damage to existing vessels and possible failure of repair.

The frequency of circulating endothelial cells (CECs) in healthy blood is about 0.5-2 cells/ml of whole blood. The basal level of endothelial turnover is low, however, acute stress injury of the vascular endothelium causes an increase in CEC of up to

10 fold or more (Blann *et al.*, 2005; Dignat-George and Sampol, 2000). Damage of the endothelium results in EC death causing loss of the antithrombotic properties of the vessel wall and enhances the number of circulating ECs.

CECs have been defined based on their morphology, their von Willebrand factor intra-cellular staining and by the expression of CD146 (also known as S-endo). Characterisation of CECs in a clinical setting has been performed by several groups but has failed to further clarify their phenotype. Makin *et al.*, (2004) failed to identify any CD146 CECs that co-stained with CD34. In contrast, Del Papa *et al.*, (2004) and Mancuso *et al.* 2001 defined CECs by the co-expression of CD146 and CD34, whilst Zhang *et al.*, 2005 actually defined their CECs as those MNCs simultaneously expressing CD105, CD146 and CD34. Almost all reports identified a rise in numbers of CEC in a variety of pathological conditions and that this correlated with disease severity and risk factors (Blann, 2006). Therefore, detection of CECs could be used as a biomarker for predicting the presence and severity of vascular diseases (Dignat-George and Sampol, 2000). Indeed the number of CECs may serve as diagnostic or prognostic parameters of vascular injury and tumour growth (Hunting *et al.*, 2005).

The hypothesis that cells in the adult circulation could proliferate, migrate and contribute to growth of new endothelium and blood vessels dates back to (1963), when Stump *et al.* suggested that new endothelium on the flow surface of the graft was derived from blood borne cells. This was termed fallout healing and it was unknown if it represented mature endothelial cells that detached from the mature vascular wall or whether they were circulating endothelial cells. Fully differentiated endothelial cells migrate to tumours and sites of injury from neighbouring blood vessels (Hanahan *et al.*, 1996, Holash *et al.*, 1999). However mature ECs are terminally differentiated cells with a low proliferative potential and their capacity to substitute damaged endothelium is limited. Therefore the repair may require the support of other cell types and over the past few years it has become evident that circulating EPCs were the responsible for that.

5.1.8 Endothelial growth factors

5.1.8.1 VEGF

VEGF is a homodimeric heparin-binding glycoprotein, an endothelial cell-specific mitogen and survival factor, produced by most cell types. VEGF is an essential determinant of hemangioblast differentiation into endothelial progenitor cells or angioblasts, and haematopoietic stem cells (Risau and Flamme, 1995). It is possible that VEGF may have a similar complementary function in adults to promote vasculogenesis (Asahara *et al. (b)*, 1999). In fact, VEGF has been shown to induce

mobilisation of bone marrow derived EPCs and result in increased differentiation of EPCs *in vitro* and to augment corneal neovascularisation *in vivo* (Asahara *et al. (b)*, 1999).

Members of the vascular endothelial growth factor (VEGF) family include five structurally related members, VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF). VEGF-A acts mainly through the tyrosine kinase receptor 2 VEGFR2 (also known as kinase insert domain receptor (KDR), human homologue; or Flk-1 murine homologue). VEGFR2 is a crucial receptor for transmitting cellular signals for the proliferation, differentiation and migration of endothelial cells.

VEGF-B and PlGF only bind and activate VEGFR-1 (Flt-1). VEGFR1 might be more important for vascular remodelling than for conveying signals essential for angiogenesis and haematopoiesis (Neufeld *et al.*, 1999). VEGF-C and VEGF-D bind to both VEGFR2 and VEGFR3, and mediate angiogenic and lymphatic neovascularisation signals, respectively (Cao, 2005). VEGF-C is also expressed in various human cancers (Salven *et al.*, 1998) and has a potent angiogenic effect *in vivo* (Stacker *et al.*, 2002, Cao *et al.*, 1998). The role of VEGF and VEGFR2 in haematopoiesis and angiogenesis is further supported by conventional gene knockouts experiments, which resulted in early embryonic lethally owing to impaired haematopoiesis and angiogenesis (Shalaby *et al.*, 1995). The blocking of VEGFR-1 with monoclonal antibodies successfully attenuated blood vessel formation (Autiero *et al.*, 2003).

PlGFs are members of a family that consists of PDGF-A,-B,-C and –D which are structurally related to the VEGFs (Bergsten *et al.*, 2001). PDGF-BB seems to be the most promising pro-neovascularisation candidate, improving perfusion as well as function in preclinical models of myocardium ischaemia (Post, 2002) inducing collateralisation (Martins *et al.*, 1994) and recruiting pericytes (Hirschi *et al.*, 1999). PDGF-CC has also been reported to be angiogenic (Cao *et al.*, 2002).

Alternatively, it has been reported that VEGFR2 induces tyrosine phosphorylation of the endothelial adherence junction components such as VE-cadherin (Esser *et al.*, 1998). Cadherins constitute a large family of membrane receptors involved in calcium dependent homotypic cell-cell interactions. Endothelial cells contain two types of cadherins; VE-cadherin, which is only found at endothelial adherence junctions and N-cadherin, which is diffusely localised over the whole cell surface (Angst *et al.*, 2001). Absence of VE-cadherin leads to embryonic lethality at gestation day 9.5 in mice because of calcium insufficiency (Carmeliet *et al.*, 1999). Several relationships between VE-cadherin and VEGFR2 have been reported recently. Carmeliet *et al.*, found that VEGF-dependent survival was dependent on expression

of VE-cadherin and Calera *et al.*, (2004) proposed that VE-cadherin has an essential role in maintaining the steady-state level of VEGFR2. Monocytes are thought to be positive for VEGFR1 (Flt-1) but negative for the other two receptors of VEGF, VEGFR-2 and –3 (Schmeisser *et al.*, 2001).

To date there is little information on the endogenous regulation of growth factors during conditions that favour neovascularisation The best described so far is the hypoxia response system which is dependent on the oxygen tension. Hypoxia-inducible factor 1 (HIF-1) induces transcription of angiogenic target genes such as activation of VEGF gene expression (Kelly *et al.*, 2003, Cao, 2005).

5.1.8.2 Fibroblast growth factors

Fibroblast growth factors (FGFs) have been shown to play an important role in modulating vascular responses by increasing endothelial cell proliferation.

5.1.8.3 Insulin like growth factor

In vivo information on the angiogenic potency of insulin like growth factor (IGF-1) is limited. IGF-1 is both cardioprotective against ischaemia and angiogenic (Su *et al.*, 2003), but concerns about aggrevation of diabetic retinopathy and coronary artery disease (Delafontaine *et al.*, 2004) have forestalled further clinical development (Cao, 2005).

5.1.8.4 Hepatocyte growth factor

Hepatocyte growth factor (HGF) induces EPC mobilisation from the bone marrow and enhances the proliferation of endothelial cells *in vivo* (Ishizawa *et al.*, 2004). There is growing evidence that protein and gene therapy with HGF improves blood flow in various hindlimb (Morishita *et al.*, 1999) and cardiac models (Ahmet *et al.*, 2003) of ischaemia. HGF/SF is currently in phase I trials in patients with peripheral artery disease (PAD). Other growth factors are under study such as ascorbic acid, which was shown to induce cardiac differentiation in ES cells (Takahashi *et al.*, 2003).

It is probable that *in vivo* most of the growth factors secondarily induce other factors that are required to complete specialised tasks such as pericyte recruitment and it is not known if a single growth factor could control a process so complex (Cao, 2005). Numerous studies have shown that tumour growth and diabetic retinopathy can be dependent on angiogenesis and angiogenic growth factors (for review see Folkman, 2002; Witmer *et al.*, 2003). However, little evidence exists as to how exogenous growth factors actually simulate tumour growth and diabetic retinopathy (Cao, 2005).

Urbich *et al. (a)*, (2005) compared the expression of potential angiogenic growth factors by EPC and HUVEC cells. They found that the expression of and release of growth factors such as VEGF-A, SDF-1, IGF-1 and HGF was higher in EPC by comparison with HUVEC (mature endothelial cell line). Factors which can support the survival and function of tissue residing cells in a paracrine manner, thereby accelerating the process of new blood vessel formation and regeneration of ischaemic tissues have been described as pro-angiogenic growth factors (Urbich *et al. (a)*, 2005).

5.1.9 *Ex vivo* manipulation of EPC.

The ability to culture endothelial cells or endothelial-like cells from different sources is important, since these rare cells can now be isolated and expanded for further study. Isolation methods include the use of adherence to isolate cell subsets, the use of specific monoclonal antibodies conjugated to magnetic microbeads or fluorochrome to isolate specific cell populations by MACS or FACS respectively from mononuclear cell pools of mobilised peripheral blood, cord blood or bone marrow (Hristov *et al.* (a), 2003). Isolated putative EPCs are cultured in medium with specific growth factors in order to expand and drive them to endothelial lineage cells. The incubation *in vitro* with a mixture of growth factors, the adhesion on specific substrates (e.g. on fibronectin, collagen or gelatin), or the contact with extracellular matrix or with different cell types will influence their proliferation and differentiation (Hristov *et al.* (a), 2003).

To date most of the experimental approaches to select EPCs are based on the plating of low-density mononuclear cells on fibronectin-coated surfaces and growth in the presence of endothelial growth factors such as VEGF (Vasa *et al.*, 2001). Typically, adherent cells have been classified as primary EPCs after 3-7 days on the basis of endothelial-like morphology, endothelial markers, lectin binding, and uptake of acetylated low-density lipoproteins or formation of tube-like structures on Matrigel. (Walenta *et al.*, 2005; Vasa *et al.*, 2001). Alternatively, MNCs have been pre-selected for either CD34 or CD133 positive cells and then characterised by surface markers. Although enriched $CD34^+$ or $CD133^+$ progenitors usually in the presence mostly of VEGF can generate endothelial cells and exhibit revascularisation *in vivo*, these cells represent a very small subset of the MNCs and only a minimal number of cells can be isolated from mobilised blood, cord blood or bone marrow (Walenta *et al.*, 2005). However, if adherence related selection of cultured MNCs allows the recovery of sufficient EPC numbers for therapeutic treatments.

Lin *et al.*, (2000) defined outgrowth endothelial cells (OEC), which developed after

3 weeks in culture. Typically, they are endothelial cells with an extraordinary growth capacity providing compelling evidence for a stem or progenitor origin. Gulati *et al.*, (2003) examined the relationship between angiogenic function of early endothelial progenitor cells (EPCs) and late outgrowth endothelial cells in culture (OECs). PBMNCs cultured for 7 days generated adherent spindle-shaped cells which expressed endothelial markers and were strongly positive for the monocyte marker CD14. When MNC were maintained in culture they produced proliferating OEC colonies at 2 to 3 weeks (Gulati *et al.*, 2003). The OECs were CD14-negative cells (probably $CD133^+$-derived), had more pronounced endothelial morphology and proliferated up to 20 passages. Critically OEC never developed from $CD14^+$ cultures thus $CD14^+$ EPC are not the precursors of OEC (Gulati *et al.*, 2003).

Kaushal *et al.*, 2001 demonstrated that seeding of the cellularised vascular grafts with ovine OECs dramatically improved graft .

The mechanisms by which these different EPC populations contribute to new vessel growth may differ. OEC and their progeny may integrate into growing endothelium, whereas EPCs with monocytic features could enhance the angiogenic process in general via release of paracrine signals, namely growth factors or cytokines (VEGF, MCP-1, bFGF, IL-6) (Rookmaaker *et al.*, 2003).

Alternatively, others in an attempt to avoid possible monocyte and mature endothelial cell contamination in EPC cultures, used a preplating step of 24-48 hours on a fibronectin coated surface. This step removes all adherent low-density MNCs and the remaining non-adherent cells are replated again (Hill *et al.*, 2003). Fulkes SM *et al.* (from Stem Cell Technologies Inc report) showed that 33.1±5.3% mononuclear cells including $CD14^+$ and mature endothelial cells ($CD105^+CD45^-$) were removed after incubation for 48h on fibronectin-coated plates.

Further research is required to identify optimal culture conditions for *ex vivo* expansion of EPC, and for standardization of the procedures used for their isolation, phenotypic characterization, and evaluation of their ability to re-endotheliase the damaged endothelial areas. Identifying culture conditions that will allow EPC to expand and/or differentiate is very important for their clinical application.

5.1.10 Mesenchymal cells as a source of endothelial cells

Non-haematopoietic mesenchymal stem cells (MSCs) were discovered by Friedenstein, 1976. They were described as plastic adherent stromal cells from bone marrow capable of differentiating into osteoblasts, adipocytes and chondrocytes. More recently MSCs have been recovered from other tissues such as adipose tissue, fetal liver, blood, lung and cord blood (reviewed in Le Blanc and Pittenger, 2005),

and differentiated into numerous tissue lineages including endothelial, smooth muscle, skeletal myoblasts, hepatocytes, neural, and cardiomyocytes (Pittenger and Martin, 2004).

Panepucci *et al.*, 2004 compared MSCs isolated from cord blood with MSCs from bone marrow and showed that both were functionally similar, shared most of the expressed transcripts with the exception of some set of genes which may reflect differences related to their origin. Genes related to antimicrobial activity and to osteogenesis were predominantly expressed in BM-MSC, whereas higher expression of genes which participate in matrix remodelling and angiogenesis were mainly expressed in CB-MSCs.

There is a need for a quantitative assay to assess MSCs in a given population, because there is no single marker or combination of markers that specifically identifies MSCs. Controversy still exists over the *in vivo* phenotype of MSC. However, *ex vivo* expanded MSCs are mostly defined by their high proliferative capacity and multipotentiallity, their lack of expression of haematopoietic markers such as CD34, CD45 or CD14 (Pittenger *et al.*, 1999) and their positivity for CD73 (SH3), CD105 (SH2), CD166, CD90 and CD29 markers (Pittenger *et al.*, 1999; Barry *et al.*, 2001; Deans and Moseley, 2000).

MSCs have a cell surface phenotype that is poorly immunogenic (Pittenger *et al.*, 1999) and one remarkable aspect of MSC physiology is that may actually suppress inflammation and immunological responses (Gregory *et al.*, 2005). In fact, these immunomodulatory properties, with the inhibition of many T-cell interactions, are probably explained by their lack of expression of HLA type II receptor and co-stimulatory molecules such as B7-1 (CD80), B7-2 (CD86) and CD40 necessary for instigation of T cell proliferation (Di Nicola *et al.*, 2002; Tse *et al.*, 2003; Krampera *et al.*, 2003; Angoulvant *et al.*, 2004; Le Blanc *et al.*, 2003; Bartholomew *et al.*, 2002). MSCs express MHC I, but class II molecules are seen at only a very low level (Zimmet and Hare, 2005). Despite the increase in MHC II expression following differentiation, MSCs did not show increased T cell interaction (Le Blanc *et al.*, 2003).

The way in which MSCs suppress T-cell activation and modulate the immune response has not been yet resolved. However, several mechanisms have been proposed. Suppression seems to be mediated by a soluble factor or factors produced by MSCs, because suppression still occurs if MSCs and lymphocytes are separated in a transwell system (Rasmusson *et al.*, 2003; Di Nicola *et al.*, 2002; Tse *et al.*, 2003).

There is an urgent need for better treatment and prevention of graft versus host disease (GvHD) after autologous stem cell transplantation. Thus, in theory, allogeneic MSCs could have an apparent clinical advantage (Silva *et al.*, 2005). Bartholomew

et al., (2002) showed this immunosuppressive effect of MSCs *in vivo*, in which infusion of *ex vivo* expanded matched donor MSCs delayed the time to rejection of histoincompatible skin grafts in a baboon model. Silva *et al.*, (2005) also showed that implantation of MSCs is safe an effective. In addition to their immunomodulatory properties in allogeneic transplantation, MSCs could be very useful for the treatment of diseases of mesenchymal lineage tissues e.g. MSC differentiated to osteoblasts could be used as an effective therapy for osteogenesis imperfecta (Le Blanc and Pittenger, 2005).

Moreover, MSCs have been infused into the infarcted heart and showed improved recovery. Gojo *et al.*, (2003) showed that MSCs were able to differentiate into cardiomyocytes, endothelial cells and smooth muscle cells by direct injection into adult heart. MSCs did not transform into malignant cells or form excess extracellular matrix. Therefore MSCs may supply an ideal donor source of cardiovascular cells. Similarly, Nagaya *et al.*, (2005) showed that MSCs differentiated into a variety of cells, including beating cardiomyocytes and vascular endothelial cells. Others have found similar positive encouraging results (Silva *et al.*, 2005; Jiang *et al.*, 2002; Strauer *et al.*, 2002). MSCs in addition to having high proliferative and self-renewal capability also secrete a broad spectrum of angiogenic cytokines, critical for maintaining lasting effects after clinical application (Tang *et al.*, 2004).

In comparative experiments, Iwase *et al.*, (2005) showed that transplantation of MSC caused greater improvement in hindlimb ischaemia than did transplantation of MNC. Compared to MNCs, MSCs survived well and differentiated into endothelial cells, and also to vascular smooth much cells. Moreover perfusion recovery of $1x10^6$ MSC transplantation was equivalent to that of $5x10^6$ MNC transplantation. MSCs secreted larger amounts of VEGF and bFGF compared with the amounts secreted by MNCs. Interestingly only MSCs secreted SDF-1, which has been shown to induce angiogenesis *in vivo* and *in vitro*.

The relationship between MSCs, $CD14^+$ cells and HSCs is not known (Romagnani *et al.*, 2005). Kuwana *et al.*, (2003) described a population of $CD14^+$ monocytes (MOMPs) that could differentiate into several distinct mesenchymal cell lineages. These cells were isolated from circulating MNCs cultured on fibronectin for 7 days and had unique molecular phenotype $CD14^+CD45^+CD34^+$. Very recently, it has been demonstrated that these cells could also differentiated into both mesodermal and neuroectodermal lineages (Kodama *et al.*, 2006).

5.1.11 MAPC as a source of endothelial cells

Preliminary evidence that MAPC may be able to generate EC was provided by Reyes and Verfaillie, (2001). These cells, possible within the MSCs, were called multipotent adult progenitor cells (MAPC), could expand for greater than 70 to 150 population doublings and differentiated not only into mesenchymal lineage cells but also into endothelium, neuroectoderm, and endoderm (Jiang *et al.*, 2002). MAPC cultured with VEGF at a high density in a serum free media were induced to endothelial lineage whereas MAPC cultured with 10% FCS differentiated into osteoblasts, chondroblasts and adipocytes (Reyes *et al.*, 2002).

5.1.12 Adipose tissue as a source of endothelial cells

For a long time, adipose tissue was considered unimportant. However, recently its endocrine functions emerged and appeared to play a key role in many physiological situations such as inflammation and immunity. During embryonic development a crucial link exists between adipose cells and the capillary network (Di Nicola *et al.*, 2002; Tse *et al.*, 2003) and recent studies have demonstrated that adipose cells can be now considered vascular progenitors. It is unknown whether these cells are MAPC derived. Martinez-Estrada O.M *et al.*, (2005) showed that it is possible to generate a large number of Flk-1$^+$ cells from adipose tissue and differentiate them into endothelial cells. Similarly, Nakagami *et al.*, 2005 showed that adipose tissue derived cells significally increased endothelial cell viability, migration and tube formation mainly through the secretion of VEGF and HGF.

Cao, (2005) identified adipose derived adult stem cells (ADAS cells) a CD31$^-$CD34$^-$ CD106$^-$Flk1$^+$ cell population from adipose tissue which can be induced to differentiate into cells of osteogenic and adipogenic lineages *in vitro*.

All of these recent results suggest that adipose tissue is another possible source of cells for autologous therapeutic angiogenesis especially as adipose tissue is very easy to obtain (Casteilla *et al.*, 2005) and because of their capacity to expand *ex vivo* (Planat-Benard *et al.*, 2004; Casteilla *et al.*, 2005).

5.1.13 Mobilisation. Modulators of EPC production

When minor new vessel formation is required in human adult life, these may arise by outgrowth from *in situ* mature endothelial cells in existing vessels (angiogenesis). However these cells are mature differentiated cells with low proliferative potential and a limited life span and are thought to be unable to incorporate into remote target sites. In a situation of larger-scale tissue repair, it is possible that quiescent EPCs, in response to an increased plasma level of certain cytokines and growth factors, may relocate to damaged areas promoting endothelial cell differentiation

(Hristov *et al.* (b), 2003; Murohara, 2001). However, if this does occur naturally, for example in infracted hearts or ischaemic diabetic limbs, it is clear that the effect is not sufficient to repair the damage to any significant extent. Translocation of EPCs may be impeded by underlying vascular disease where circulation is poor due to the damaged vasculature.

The administration of angiogenic cytokines to mobilise and expand putative EPC to augment resident EPC populations in areas of endothelial damage might represent an alternative means to increase post-natal vasculogenesis. This could address the problem of endothelial dysfunction or deficiency in, for example, older, diabetic and/or hypercholesterolemic patients (Murasawa and Asahara, 2005).

It is important to consider though, that aging and certain disease situations could negatively influence the frequency of EPC mobilisation (Murasawa and Asahara, 2005). Tepper *et al.*, (2002) have shown the impairment of EPC incorporation into vascular structures in type II diabetes and Murayama and Asahara, (2001) showed that EPCs from older patients compared with those from younger patients with clinical ischaemia had significantly less therapeutic effect in rescuing ischaemic limbs of mice.

Mobilisation of stem cells in the bone marrow is determined by the local microenvironment (stem cell niche) (Papayannopoulou, 2004). Mobilising cytokines impede the interactions between stem cells and stromal cells, and finally allow them to leave the bone marrow via transendothelial migration. This is true for $CD34^+$ HSC but it is unknown for endothelial progenitor cells.

EPC mobilisation requires further study as in some models adverse effects have been described as EPCs contribute to tumour neovascularisation (Lyden *et al.*, 2001).

5.1.13.1 Tissue ischaemia

Physiological ischaemia is believed to be a potent angiogenic stimulus and the predominant signal to induce mobilisation of EPCs from the bone marrow. Ischaemia or hypoxia may increase vascular permeability, enhance the release of chemoattractant factors, and promote the expression of adhesion proteins which may facilitate the homing process (Perin *et al.*, 2003). Ischaemia thereby is believed to upregulate VEGF or SDF- 1 secretion (Lee *et al.*, 2000; Pillarisetti and Gupta, 2001), which in turn are released to the circulation and induce mobilisation of progenitor cells from bone marrow (Urbich and Dimmeler, 2004). Indeed, patients undergoing coronary artery bypass grafting (CABG) or those who have suffered extensive burns have elevated VEGF plasma levels, which promotes rapid mobilisation of endothelial cells to the peripheral blood (Gill *et al.*, 2001). However,

the increase of the level of these cytokines and growth factors seems to depend on the severity of the case. Shintani *et al.*, (2001) have reported that plasma levels of VEGF positively correlate with the number of $CD34^+$ cells in circulation.

5.1.13.2 Growth factors

Equivalent to ischaemia, exogenously added cytokines act as chemoattractants and lead to mobilisation of EPC therefore increasing the frequency of circulating EPCs (Urbich and Dimmeler, 2004). At the present it is not known which growth factor most potently elevates the number of EPCs. Vascular endothelial growth factor (VEGF) is thought to be a critical angiogenic growth factor (Carmeliet *et al.*, 1996; Ferrara and Bunting, 1996; Shalaby *et al.*, 1995). A number of studies have shown that VEGF contributes to neovascularisation by mobilising bone marrow-derived EPCs; in patients undergoing VEGF gene transfer for critical limb ischaemia (Kalka *et al.* (b), 2000) and in patients after myocardial ischaemia (Kalka *et al.* (c), 2000). VEGF165 was shown to rapidly mobilise haematopoietic stem cells and circulating endothelial precursor cells (Moore *et al.*, 2001; Hattori *et al. (a)*, 2001). However, further studies are needed to test possible side effects as some reports claimed that systemic administration of VEGF induced oedema formation (Vajanto *et al.*, 2002). Administration of stromal-derived factor-1 (SDF-1) showed similar positive angiogenic effects to VEGF whereas angiopoietin-1 induced a delayed and a less pronounced mobilisation of endothelial and haematopoietic precursors (Moore *et al.*, 2001; Hattori *et al. (a)*, 2001).

M-CSF, GM-CSF and G-CSF, initially discovered as stimulators of haematopoietic progenitor cells and myeloid cells have also been shown to mobilise EPCs from BM (Takahashi *et al.*, 1999).

Nakano, K *et al.* 2006 suggested that M-CSF could mobilise EPCs through augmenting the production of VEGF, mainly produced by myeloid lineage cells in the bone marrow, resulting in the augmentation of blood flow and the increased number of blood vessels in ischaemia-induced limbs. M-CSF administration has the advantage that it does not augment mobilisation by raising VEGF serum levels, thus avoiding the possibility of major side effects referred above (Nakano *et al.*, 2006).

GM-CSF is a relatively inexpensive and safe cytokine extensively used clinically. Cho *et al.*, (2003) showed an accelerating endothelialisation through EPC mobilisation after GM-CSF administration. It has also been reported that not only GM-CSF but also G-CSF can mobilise EPCs (Takahashi *et al.*, 1999). Takahashi *et al.*, (1999) demonstrated that G-CSF administration induced EPC mobilisation and

enhanced neovascularisation in severely ischaemic tissues as well as *de novo* corneal vascularisation.

Different authors have stated the benefits of the use of G-CSF for mobilisation of EPC (Ince *et al.*, 2005; Kong *et al.*, 2004; Yoshioka *et al.*, 2006). For example, Kong *et al.* (2004) reported that G-CSF induced mobilisation of EPCs enhanced endothelialisation of the injured artery and inhibited neointimal formation in a rat balloon injury model. Consistent with the Kong *et al.* results, Yoshioka *et al.*, (2006) showed that G-CSF pre-treatment accelerated re-endothelialisation and inhibition of neointimal formation after vascular injury. However, the effects of G-CSF on ischaemic lesions are still controversial (Kang *et al.*, 2004; Ince *et al.*, 2005). Kang *et al.* (2004) demonstrated that G-CSF promoted angiogenesis and improved cardiac function when administered in patients with coronary artery disease (CAD); however increased restenosis was observed as a serious adverse effect after percutaneous coronary intervention (PCI). There is a concern that the administration of G-SGF could accelerate arteriosclerosis and may give rise to thrombosis (Lindemann and Rumberger, 1993)

G-CSF also promotes inflammation by inducing a profound increase in the number of circulating leucocytes (Morimoto *et al.*, 1990; Adachi *et al.*, 2003). Because inflammation plays a key role for the development of atherosclerotic lesions and restenosis, as well as in plaque instability leading to acute coronary syndromes, the safety of G-CSF is still questioned.

Alternative EPC mobilisation agents with a lower pro-inflammatory profile, like EPO, statins, or exercise, could more selectively enhance EPC levels without inducing inflammation.

5.1.13.3 Erythropoietin (EPO) as an EPC mobilisation agent

Mature endothelial cells express erythropoietin (EPO) receptors (Heeschen *et al.*, 2003). Administration of the hormone EPO is known to increase proliferation and maturation of erythrocytes and to augment neovascularisation in mice (Heeschen *et al.*, 2003) and in men at least in part by enhancing EPC mobilisation from the bone marrow (Bahlmann *et al.*, 2004). The correlation between EPO serum levels and the number of $CD34^+$ or $CD133^+$ HSCs in bone marrow in patients with ischaemic coronary artery disease further supports the important role of endogenous EPO levels (Heeschen *et al.*, 2003). Hence, EPO serum levels may help in identifying patients with impaired EPC recruitment capacity (Heeschen *et al.*, 2003).

5.1.13.4 Statins as EPC mobilisation agents

Therapeutic mobilisation of EPCs has been studied not only using natural haematopoietic or angiogenic stimulants but also using anti-hypercholesterolaemia drugs. The statin class of cholesterol-lowering hydroxyl-methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitors catalyse the synthesis of mevalonate, which is a rate limiting step in the cholesterol synthesis.

Statins are not only believed to reduce cardiovascular events in patients at risk, but also stimulate endothelial cell bioactivity *in vitro* and enhance angiogenesis *in vivo* (Kureishi *et al.*, 2000). An increase of the functional activity of EPCs has been shown in mice (Dimmeler *et al.*, 2001, Llevadot *et al.*, 2001) and in patients with stable coronary artery disease (Vasa *et al.*, 2001). Statins also increase expression of adhesion molecules by EPCs that facilitate their incorporation into sites of neoangiogenesis (Walter *et al.*, 2002).

5.1.13.5 Exercise as an EPC mobilisation stimulus

The mechanisms by which physical exercise increase EPC levels are not entirely clear. Aicher *et al.*, (2005) speculated that the induction of ischaemia in the muscles enhances circulating cytokine levels and increases EPC mobilisation. Rehman *et al.*, (2004) demonstrated that only 10 minutes of exercise acutely increases the circulating EPC cell population. Steiner *et al.*, (2005) showed that exercise training augmented the number of circulating EPCs in patients with cardiovascular risk factors and coronary artery disease and is associated with improved vascular function and NO synthesis. Plasma levels of VEGF did not change in response to exercise. Exercise could be used as a complement for pharmacological interventions such as statins or G-CSF therapy. At the same time, unravelling the mechanisms of exercise-induced EPC mobilisation may result in the development of novel pharmacological pathways to improved endothelial function and enhance angiogenesis.

Although the molecular signalling pathways by which EPCs are mobilised to peripheral circulation is not confirmed, several studies indicate that is via the activation of the Pl3/Akt pathway. VEGF, EPO, oestrogens (Strehlow *et al.*, 2003; Iwakura *et al.*, 2003) and exercise (Laufs *et al.*, 2004; Adams *et al.*, 2004) all appear to exert their effect on EPCs through the Pl3K/Akt-pathway.

5.1.14 Chemotaxis: migration and invasion of circulating EPCs

It is not known which physiological or pathological factors influence the homing signals that direct circulating EPCs to sites of injured vessels (Hristov *et al.* (b), 2003). The local bone marrow microenvironment, or stem cell niche, governs the maintenance and mobilisation of bone marrow mobilisation stem cells (Calvi *et al.*, 2003; Zhang *et al.*, 2003). Mechanistically, cytokines inducing mobilisation interfere with the interactions between stem cells and bone marrow stromal cells, which allow stem cells to disengage the bone marrow, and enter the blood stream. Stem cell mobilisation is mediated by proteinases such as elastase, cathepsin G, and matrix metaloproteinases (MMPs) (Lapidot and Petit, 2002). For example, G-CSF releases proteinases (elastase, and cathepsin G) from the neutrophils and induces the release of SDF-1 by the stromal cells, forcing $CXCR4^+$ cells to leave the bone marrow. Several investigators have reported that $CD34^+$ cells express CXCR4 and that SDF-1 could induce $CD34^+$ cell migration *in vitro* (Mohle *et al.*, 1998).

Homing is mediated through complex interactions between microenvironmental endothelial/stromal cells and haematopoietic stem cells. Adhesion molecules belonging to the integrin family, CXCR4, metalloproteinases and cathepsins seem to have an important role.

5.1.14.1 Integrins in EPC homing

Integrins may be involved in the homing of progenitor cells to ischaemic tissues. (Urbich and Dimmeler, 2004). The initial step of homing of the progenitor cells to ischaemic tissues involves adhesion of progenitor cells to vascular endothelial cells activated by cytokines and ischaemia and the transmigration of the progenitor cells through the endothelial cell monolayer (Vajkoczy *et al.*, 2003).

beta-1 Integrins are capable of mediating cell-cell interactions of various cells including haematopoietic stem cells and leucocytes to extracellular matrix proteins and to endothelial cells. Cell adhesion to the extracellular matrix is an important process that controls cell migration, proliferation, survival and differentiation, and it is hypothesized that the cellular expression of adhesion molecules such as integrins and cadherins are critically important (Li *et al.*, 2005).

Integrins are heterodimeric cell-surface receptors composed of an *alfa* and *beta* subunit heterodimers, which integrate the extracellular matrix with the intracellular cytoskeleton and mediate cell adhesion, survival, differentiation, growth and migration. *beta*-1 integrin is one of the most important families of integrins mainly

because it mediates cell-cell or cell-extracellular matrix interactions. However, there is only very recent direct experimental evidence to support this suggestion. Antibody perturbation to integrin *beta*-1 significally decreased the cell survival and incorporation of freshly unexpanded $CD117^+$ cells after implantation (Li *et al.*, 2005). Moreover, Li *et al.*, (2005) showed that integrin-*beta*-1 is a critical adhesion molecule for inducing therapeutic angiogenesis by regulating survival and differentiation after implantation into ischaemic tissue. Newly isolated bone-marrow $CD117^+$ cells had the potential to induce angiogenesis but *ex vivo* expanded $CD117^+$ cells, with significant decrease of *beta*-1 integrin expression, had low angiogenic potency (Li *et al.*, 2005).

5.1.14.2 SDF-1/CXCR4 in EPC homing

Understanding the factors that attract circulating EPCs to the ischaemic sites is very important. Stromal cell-derived factor-1 (SDF-1) is a member of the chemokines CXC subfamily considered to play an important role in the trafficking of haematopoietic stem cells between the bone marrow and peripheral blood (Yamaguchi *et al.*, 2003). CXCR4, a 7-transmembrane-spanning G-protein-coupled receptor is the only known receptor for SDF-1. SDF-1/CXCR4 interaction is reported to play an important physiological role in haematopoiesis during embryogenesis (Nagasawa *et al.*, 1996), vascular development and cardiogenesis (Tachibana *et al.*, 1998). Murine bone marrow engraftment and repopulation by human SCID-repopulating stem cells are completely blocked by neutralising antibody to CXCR4, which suggests that the SDF-1/CXCR4 axis is essential for the homing of human haematopoietic stem cells (Peled *et al.*, 1999). In addition, Kortesidis *et al.*, (2005) showed that SDF-1 may play a role in the maintenance, survival and osteogenic capacity of immature bone marrow stromal stem cells.

CXCR4 is also expressed by endothelial cells (Volin *et al.*, 1998) and evidence suggests that SDF-1 could have direct affects on vasculogenesis. Tachibana *et al.*, (1998) reported that mice lacking SDF-1 had defective formation of large vessels supplying the gastrointestinal tract. Moreover Hattori *et al.*, (b) (2001) reported that plasma elevation of SDF-1 induced mobilisation of mature and immature stem cells including EPCs. Further, Yamaguchi *et al.*, (2003) showed that local administration of SDF-1 *in vivo* stimulates recruitment of EPCs to the ischaemic tissue.

5.1.14.3 Matrix Metalloproteinases (MMPs) in EPC homing

Endothelial cells are activated by and migrate toward angiogenic stimuli. In the early stages of the neo-angiogenic process the activated endothelial cells act like metastatic cancer cells. Activated endothelial cells express significant levels of matrix degrading enzymes, particularly matrix metalloproteinases (MMPs) that degrade and digest the capillary basement membrane and allow the cells to move toward an angiogenic stimulus. *In vitro* techniques designed to investigate this process are divided into migration and invasion assays.

5.1.14.4 Proteases (Elastases and Cathepsins) in EPC homing

Elastases and cathepsins are proteases, which are mainly found in the granules of neutrophils, and their biological activity involves proteolytic degradation of proteins (Ng *et al.*, 2004). Inhibition of elastase activity prevents mobilisation of bone marrow $CD34^+$ cells into peripheral blood by reducing the degradation of stroma-derived factors (SDF-1) and stimulation with G-CSF resulted in increased degradation of SFD-1.

Cathepsins are cysteine endopeptidases that belong to the family of papain-like proteolytic enzymes that are principally located in the endosomal/lysosomal compartment of the cells (Turk *et al.*, 2000).

Urbich *et al.* (b) (2005) indicated that EPCs express high levels of cathepsin. CathL deficient mice showed impaired functional recovery following hind limb ischaemia and infused CathL deficient progenitor cells neither homed to sites of ischaemia nor augmented neovascularisation. Mature endothelial cells have low levels of CathL by comparison to EPCs. Forced expression of CathL in mature endothelial cells considerably enhanced their invasive activity. Thus, it seems that cathepsin L has a critical role in the integration of circulating EPC into ischaemic tissue and is required for EPC-mediated neovascularisation and invasion.

5.1.15 Gene modified EPC therapy

Gene therapy may be an alternative strategy to address the limited quantity of EPCs in circulation even in healthy conditions. Isolated and expanded EPCs could be genetically modified for subsequent autologous re-administrated (Murasawa and Asahara, 2005). EPCs transfected with adenovirus vectors encoding for specific genes such as VEGF would therefore overexpress angiogenic growth factors for long periods of time and be able to enhance an angiogenic response (Masuda and Asahara, 2003); (Asahara *et al.* (b), 1999). Iwaguro *et al.*, (2002) demonstrated that genetically modified EPCs rescued impaired neovascularisation in an animal model

of limb ischaemia and the injected mice significally improved neovascularisation and blood flow recovery. In addition, limb necrosis and auto-amputation was significally reduced by 63.7% by comparison with controls. Moreover, Kalka *et al.* (c), (2000) showed that VEGF gene transfer *in vivo* mobilised EPCs in patients with ischaemic coronary disease.

These gene-delivery data in animal studies, although limited, proposed gene therapy as a safe approach for cell-based vascular therapies (Gulati and Simari, 2004). However, more clinical experience is needed to resolve safety concerns as some recent studies showed angioma formation after gene delivery (Carmeliet, 2000; Lee *et al.*, 2000).

5.1.16 Smooth muscle cell interactions with EPCs

Endothelial cells can initiate but not complete angiogenesis. This maturation requires communication between ECs and supporting cells such as smooth muscle cells (SMCs) and pericytes. In fact, once the primitive EC tubes are formed, the endothelium secretes factors that lead to the recruitment of SMCs (Le Ricousse-Roussanne *et al.*, 2004). It is unknown whether EPC and SMC can be generated from a common progenitor. Pesce *et al.*, (2003) showed that $CD34^+$ cord blood cells injected into an ischaemic adductor muscles gave rise to endothelial and to skeletal muscle cells in mice. Similarly, Le Ricousse-Roussanne *et al.*, (2004) showed that both ECs and SMCs progenitors are present in cord blood, which can differentiate into both mature ECs and SMCs. *In vitro* these two cell types collaborate with each other in a three-dimensional culture to form vascular-like structures and to home to the ischaemic site.

Sata *et al.*, (2002) suggested that when endothelium is injured, circulating EPCs may adhere to the underlying smooth muscle cells and contribute to the neointimal formation by differentiation into smooth muscle cells.

Another potential source of SMCs may involve bone-marrow derived mesenchymal stem cells, which have the potential to differentiate into smooth muscle-like cells and may be recruited into the graft from the circulation (Shimizu *et al.*, 2001).

5.1.17 Therapeutic angiogenesis

Amputation of limbs in patients with diabetic foot ulcers and arteriosclerosis obliterans is a very serious problem. Vasodilators are the first choice of treatment, but this is not always effective in preventing amputation (Kawamura *et al.*, 2005). Cell regenerative therapy could develop to be more effective than surgical treatments (Kawamura *et al.*, 2005). EPC cell transplantation not only improved

neovascularisation and blood flow recovery, but notably also important biological consequences such as limb necrosis and autoamputation (Kalka *et al.* (a), 2000; Kawamura *et al.*, 2005). Augmentation of vasculogenesis was detected in a hindlimb ischaemia rat model after *ex vivo* $CD34^+$ cell injection (Murohara *et al.*, 2000). Also, Schatteman *et al.*, (2000) showed an increase in the restoration of limb blood flow in diabetic nude mice with hindlimb ischaemia after $CD34^+$ *ex vivo*-expanded cells were injected.

Ischaemic heart disease is a leading cause of morbidity and mortality in the Western world (Annex and Simons, 2005). Currently there are a large number of invasive and non-invasive treatment options for patients with coronary artery disease. Mechanical revascularisation techniques such as coronary bypass surgery and angioplasty restore flow to the compromised myocardium. Therapeutic agents such as nitrates and *beta*-blockers restore the perfusion supply/demand balance by reducing myocardial oxygen requirements. Finally cholesterol-lowering drugs influence further progression. However, after nearly a decade of trials, the development of drugs capable of stimulating revascularisation remains an exciting but unrealised goal in cardiovascular therapeutics (Annex and Simons, 2005). At the time of end-stage heart failure, the effect of drug treatment is limited, and cardiac transplantation is the only viable alternative. However, this strategy is costly and severely limited by the availability of donor hearts. In addition complications arising from the use of immunosuppressive agents may diminish the patient's quality of life (Davani *et al.*, 2005). Cell-based therapy has been the recent focus of attention for repairing injured organs, and the induction of therapeutic angiogenesis by cell implantation is a promising treatment option for ischaemic diseases.

The benefits of transplanting autologous EPCs for increasing tissue reperfusion have been established in multiple studies of experimentally induced acute or chronic ischaemia of heart or limbs (reviewed in Rafii and Lyden , 2003). *In vivo* preclinical data showed that infusion or injection of adult EPCs after myocardial infarction resulted in profound and sustained improvement of cardiac function (Kocher *et al.*, 2001; Fuchs *et al.*, 2003; Kawamoto *et al.*, 2001) (see table 5.2). Kocher *et al.*, (2001) injected $2x10^6$ DiL-labeld G-CSF mobilised adult-human $CD34^+$ cells (>98% purity) from a single donor in the tail vein of rats and within 48h of left anterior descending (LAD) coronary artery ligation resulted in new blood vessel formation and proliferation of pre-existing vasculature. 20-25% of capillaries were from human origin and they were exclusively located within the central infarct zone. Measurements showed improvements by an increase of capillary density in the infarct bed, decreased apoptosis of hypertrophied myocytes in the peri-infarct zone,

salvage and survival of the viable myocardium, inhibition of fibrosis, and improvement of left ventricular function (Zisch, 2004).

Early data obtained in human pilot trials of myocardial infarction (Assmus *et al.*, 2002; Britten *et al.*, 2003; Stamm *et al.*, 2003), advanced coronary artery disease (Fuchs *et al.*, 2003) or leg ischaemia (Tateishi-Yuyama *et al.*, 2002; Higashi *et al.*, 2004; Kawamura *et al.*, 2005) (see table 5.2) suggest that cell-based reperfusion therapy is both safe and feasible and capable of achieving revascularisation (Zisch, 2004).

Human adult EPCs have been isolated from bone marrow aspirates, peripheral blood, and cord blood. EPCs isolates re-introduced by injection or infusion into heart or limb have been shown to maintain their ability to participate in endothelial/ vessel growth at sites of ischaemic or vessel injury. Especially locally grafted EPCs showed positive effects for improving regional blood flow supply in ischaemic heart and peripheral disease, inhibiting neointimal hyperplasia by growing new endothelium in denuded arteries after balloon angioplasty (Rafii and Lyden, 2003). Taken together experimental studies performed so far by cell therapy generally showed that improvement of neovascularisation is not exclusive to one cell type because a variety of progenitors accomplish this (Davani *et al.*, 2005). However, further studies are required to choose therapeutically which most successful kind of bone marrow cells (global unfractioned bone marrow cells, or specifically selected subfractions, as isolated cells fractions containing $CD34^+$, $CD133^+$, MSC) are the most suitable cell populations for transplantation (Strauer and Kornowski, 2003).

Direct comparison of *ex vivo* expanded circulating progenitor cells peripheral blood derived and bone marrow-derived EPCs in the TOPCARE-AMI trial (transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction) (Assmus *et al.*, 2002; Britten *et al.*, 2003) showed that both preparations to be comparably effective in improving contractility and viability of infarcted myocardial segments. By contrast, in the leg ischaemia trial (Tateishi-Yuyama *et al.*, 2002), a superior impact of MNCs from bone marrow compared with peripheral blood was observed. Whether bone marrow is a richer source of EPC or not is unknown. Tateishi-Yuyama *et al.* did not use *in vitro* expanded peripheral blood MNCs whereas Assmus *et al.* did. Therefore, the inferior impact on neovascularisation detected in peripheral blood mononuclear cells compared to bone marrow in Tateishi-Yuyama *et al.* study could be due to the lack of expansion of these MNCs on fibronectin.

To date publications regarding adverse effects in experiment studies have been relatively rare (Davani *et al.*, 2005). However, the development of microinfarction has been reported when mesenchymal stem cells (MSCs) were infused directly into

a dog's coronary artery (Vulliet *et al.*, 2004). Moreover adverse calcification has been shown to be a problem in a rat model in which 28% of the rats receiving total bone marrow cells revealed some myocardial calcifications (Yoon *et al.*, 2004).

Further optimisation of EPC-based reperfusion therapy may involve multiple experimental parameters (e.g. the number of cells to inject, the placement and the number of injections, cell engraftibility and viability at the target site). The efficacy and the safety of this approach must also be established.

In current human studies of autologous mononuclear bone marrow cells, the number of cells believed to be necessary to achieve an optimal effect is of 10 to $40x10^6$ (Perin *et al.*, 2003). The cell responsible for this angiogenesis is not known but if we use $CD34^+$ as the cell required for transplantation, the optimal dose of $CD34^+$ cells for endothelial stem cell therapy remains undefined. Studies by Wollert and Drexler, (2004) have used doses ranging from 10^6-10^8 cells. The optimum dose for haematopoietic stem-cell therapy following myeloablative chemotherapy is $>2x10^6$ $CD34^+$ cells per Kg bodyweight (Siena et al., 2000). However, the dose requirement in myocardial infarction will probably not be as high as that needed in HSC therapy, because of the treatment territory involved (heart v bone marrow) and direct intracoronary injection or infusion to the targeted ischaemic or infarcted myocardium. However, further experiments are required to explore the efficacy of different doses (Perin *et al.*, 2003).

Kalka *et al. (a)*, (2000) suggested that in their animal studies heterologous transplantation required systemic injection of $0.5\text{-}2x10^4$ human EPCs/g body weight of the recipient animal to achieve satisfactory reperfusion of the ischaemic hindlimb. Rough extrapolation of this data to human suggest that a blood volume of as much as 12L may be necessary to obtain adequate numbers of EPCs to treat critical limb ischaemia in patients, therefore the shortage of the EPCs in circulation combined with their possible functional impairment associated with aging, diabetes, hypercholesterolemia constitutes a major limitation of primary EPC transplantation (Masuda and Asahara, 2003; Vasa *et al.*, 2001; Tepper *et al.*, 2002).

Local instead of systemic delivery of EPCs could reduce the dosage of cells needed. Therefore targeted and regional administration of transplantation of cells should be preferred (Strauer and Kornowski, 2003). Local co-injection of angiogenic chemokines, such as SDF-1 or VEGF, into the target area together with EPC transplantation could also augment target-specific accumulation of EPCs and increase capillary density (Yamaguchi *et al.*, 2003). Other devices utilising EPCs for clinical application such as artificial grafts first seeded with autologous cells and then implanted into the ischaemia sites were found to have increased surface

endothelisation and vascularisation when compared with the controls (Bhattacharya *et al.*, 2000). When cultured autologous ovine EPCs were seeded onto carotid interposition grafts, the EPC seeded grafts achieved physiologic motility and remained patent for 130 days versus 15 days using a non-seeded graft (Kaushal *et al.*, 2001). Alternatively, cardiomyocytes cell sheets may be effective for the improvement of cardiac function in damaged hearts (Shimizu *et al.*, 2002). Another approach to promote engraftment involves the genetic modification of EPCs to overexpress factors such as VEGF (Iwaguro *et al.*, 2002) or factors that specifically increase their biological activity and viability (e.g. human telomerase reverse transcriptase (Murasawa *et al.*, 2002) or Akt; reviewed in (Alessandri *et al.*, 2004)). Another important clinical problem will be the identification and localisation of transplanted autologous stem cells within the injured area to resolve the long-term fate of transplanted stem cells in the recipient tissue (Strauer and Kornowski, 2003). For clinical detection of stem cells, myocardial biopsies in humans hardly will be justifiable therefore, magnetic labelling and *in vivo* tracking of bone marrow cells by the use of magnetodendrimers or radioactive detection methods may be useful (Strauer and Kornowski, 2003).

EPC source	Target	Species	Injection	Reference
***In vivo* (preclinical)**				
ex vivo expanded peripheral blood MNCs	myocardial infarct	rat	Intravenous	Kawamoto *et al*, 2001
ex vivo expanded peripheral blood MNCs	myocardial infarct	rat	myocardial	Kawamoto *et al*, 2003
CD34+ G-CSF mobilised peripheral blood	myocardial infarct	rat	Tail vein	Kocher et al, 2001
ex vivo expanded CD34+MNCs cord blood	hindlimb ischeamia	rat	leg muscle	Murohara *et al*, 2000
ex vivo expanded CD34+MNCs peripheral blood	hindlimb ischeamia	mice	leg muscle	Scatteman *et al*, 2000
BM MNCs	myocardial infarct/hindlimb ischeamia	mice	Intravenous	Asahara *et al*, 1999
Lin-c-Kit+	myocardial infarct	mice	SCF and G-CSF mobilisation	Orlic *et al,* 2001
ex vivo expanded peripheral blood MNCs	hindlimb ischeamia	mice	Intravenous	Urbich *et al*, 2003
ex vivo expanded peripheral blood MNCs	hindlimb ischeamia	mice	Intravenous	Yamaguchi *et al*, 2003
MNCs spleen	Denuded carotid artery	mice	Intravenous	Werner *et al*, 2003
***In vivo* (clinical)**				
BM MNCs/ PB MNCs	ischeamic leg	human	leg muscle	Tateishi-Yuyama *et al*, 2002
BM MNCs	ischeamic leg	human	leg muscle	Higashi *et al*, 2004
G-CSF MB PBSC	ischeamic leg	human	leg muscle	Kawamura *et al,* 2005
G-CSF ex vivo expanded MB PBSC	ischeamic leg	human	leg muscle	Lenk *et al*, 2005
unfractionated BM/ ex vivo expanded PB	myocardial infarct	human	intracoronary infusion	Assmus *et al*, 2002
unfractionated BM/ ex vivo expanded PB	myocardial infarct	human	intracoronary infusion	Britten *et al,* 2003
unfractionated BM	Chronic ischemic heart failure	human	transendocardial injection	Fuchs *et al*, 2003
CD133+ BM cells	Chronic ischemic heart failure	human	intramyocardial injection	Stamm *et al*, 2003
ex vivo expanded BM cells	Chronic ischemic heart failure	human	intracoronary	Strauer *et al*, 2002
BM MNCs	Chronic ischemic heart failure	human	transendocardial	Tse *et al*, 2003
BM MNCs	myocardial infarct	human	transendocardial injection	Perin *et al*, 2003
BM MNCs	myocardial infarct	human	intracoronary	Wollert *et al*, 2004
G-CSF MB PBSC	myocardial infarct	human	intracoronary	Kang *et al*, 2004
unfractionated BM/ CD34+ BM cells	myocardial infarct	human	intracoronary	Hofman *et al*, 2005

Table 5.2

Examples of preclinical and clinical explorations into EPC-based reperfusion of tissue ischaemia or injured endothelium.

Mononuclear cells (MNC), Bone marrow (BM), peripheral blood (PB), mobilised blood (MB).

5.1.18 Endothelial Cell Lines

Endothelial cell lines have been standards for cell-based assays in the field of angiogenesis research, in anti-angiogenic drug discovery and for physiological and pharmacological investigations, such as macromolecule transport, blood coagulation, and fibrinolysis. The major part of the knowledge of endothelial cell functions comes from *in vitro* experiments with HUVECs (human umbilical vein endothelial cells) (Jaffe *et al.*, 1973). HUVECs are cryopreserved at the end of primary culture and can be cultured and propagated at least 16 population doublings. This implies that no long-term *in vitro* experiments can be performed with HUVEC (Bouis *et al.*, 2001). Typical endothelial characteristics can be divided into phenotype and function. Routine characterisation of HUVECs includes morphological and phenotype observations through serial passages. Mature endothelial cells contain Weibel-Palade-bodies, which store large amounts of von Willebrand factor (vWf). Moreover, HUVECs are known to express adhesion molecules including PECAM, ELAM, ICAM, V-CAM, E-selectin and VE-cadherin (Cambrex, technical sheet, UK). Functionally, HUVECs bind *ulex europaeus* lectin agglutinin I, take up acetylated low-density lipoproteins (ac-LDL), and present angiotensin-converting enzyme (ACE)-activity.

The isolation of primary HUVEC is laborious and experimental results obtained with different HUVEC isolates cannot easily be compared to each other because of their different donor origin (Bouis *et al.*, 2001). However, in recent years the use of primary cells has become more attractive as there is a vast and still growing range of commercially available primary ECs from almost any vascular origin. Commercial primary EC circumvent the problems of laborious EC isolation and it is possible to purchase ECs from the same original batch at a later moment if further experiments are required (Bouis *et al.*, 2001).

As an alternative, immortalised well-characterised EC lines obtained by transfection, infection or fusion with an immortal cell are generally better characterized and more stable in their endothelial traits than primary endothelial cell lines.

Presently the best-characterized immortalised macro-EC line is EA.hy926 (Bouis *et al.*, 2001). EA.hy 926 was generated in 1983 by fusion of HUVEC with human lung carcinoma cell line A549 (Lieber *et al.*, 1976). Stably presenting endothelial properties and with an extended life span, EA.hy 926 was able to substitute for secondary cultures of human umbilical vein endothelial cells (HUVEC) in a leucocyte/ endothelial adherent assay (Brown *et al.*, 1993). This hybrid cell line had adhesive properties similar to HUVEC and was shown to be beneficial for the study of factors that govern leucocyte-endothelial cell interactions (Brown *et al.*, 1993). EA.hy 926

was also a useful cell line to identify the fibrinolytic characteristics in cultured endothelial cells (Emeis and Edgell, 1988). Moreover, Pech-Amsellem *et al.*, (1996) concluded that EA.hy 926 cells were a good model for investigating endothelial cell-induced modifications of low density Lipoproteins (LDL).
Different studies require different properties of EC and it is the aim of each investigator to decide which cell line matches his or her research goal best. In our study we used both HUVEC and EA.hy 926 cell lines as a positive endothelial control to test our endothelial phenotypic and functional assays (5.2.1).

Specific studies performed in this Chapter 5

In this Chapter 5 we first investigate (5.2.2) the presence of the currently proposed EPC phenotypes in the most common and practicable potential EPC sources such as bone marrow, cord blood, G-CSF mobilised blood and normal peripheral blood aiming to conclude which phenotype is the most consistent to use as an EPC definition. To elucidate which source has the greatest potential to generate EPC and again which cell phenotype was the closest to define a true EPC population, an *in vitro* functional EPC colony assay (CFU-EPC) was used. Measurement of CFU-EPC is able to identify very low frequencies of such cells which would be difficult to quantify by flow cytometry. In the second part of this results (5.2.3) we examine which subpopulation is associated with the CFU-EPC potential by enriching different cell fractions and assessing their colony forming capacity. Different sources gave different results in CFU-EPC formation, thus we investigate further the origins of these differences.

5.2 Results

5.2.1 Preliminary studies

5.2.1 (a) Mature endothelial cells

5.2.1.1 Immunophenotype characterisation of human umbilical vein endothelial cells (HUVEC).

These cells were strongly positive for PECAM (CD31) (88.3%), *ulex europaeus* lectin agglutinin I (UEA-1) (78.42%), CD146 (71.28%) and CD29 (75.48%) markers. Moreover, these cells were positive for VEGFR2 (23%), VE-cadherin (73%), CD63 (41.6%), CD105 (61.68%) though were negative for CD45, CXCR4 and CD133 markers (Figure 5.1). Although these results are expressed as percentages of the population not overlapping the unstained negative control, the peaks were single and generally homogeneous and appear to represent general expression (or lack of expression) by all cells rather than expression only by a subpopulation of cells.

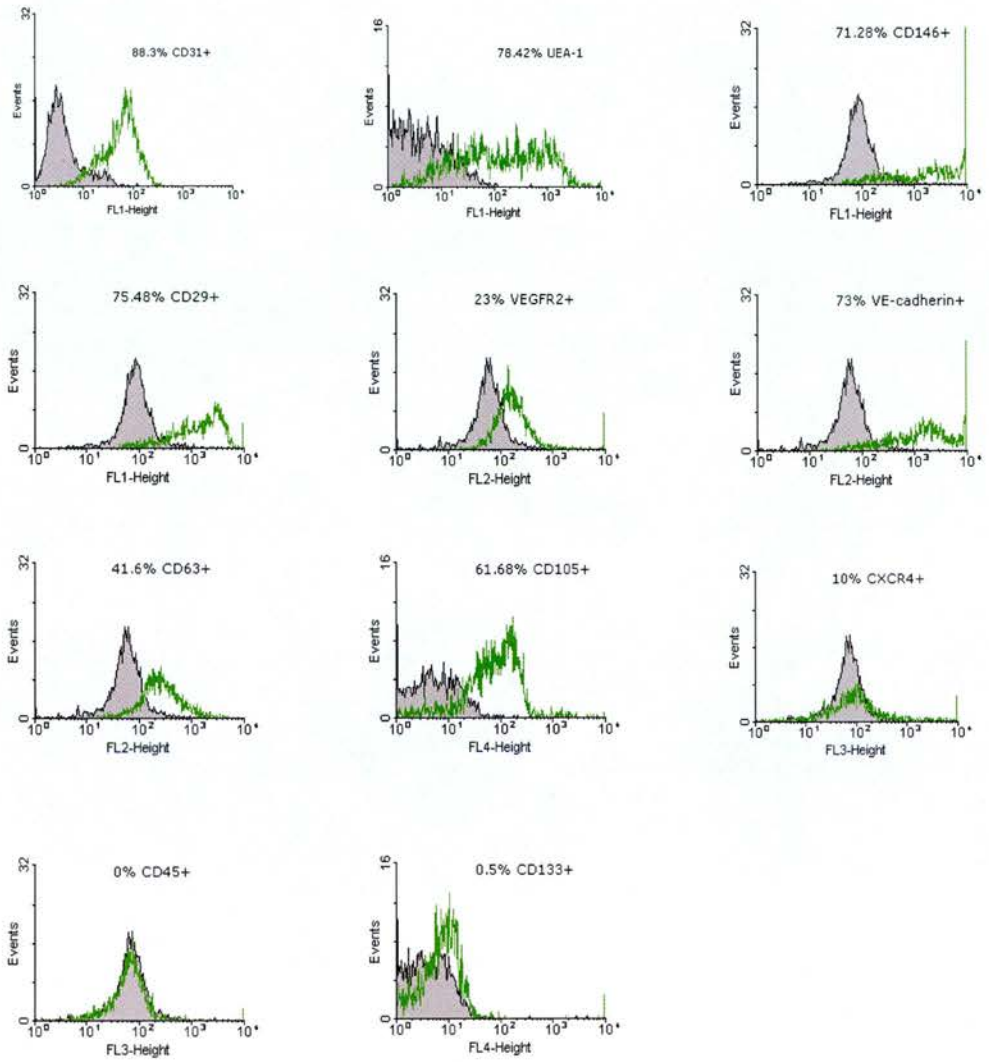


Figure 5.1 Flow cytometry analysis of cultured human umbilical vein endothelial cells (HUVEC)

Flow cytometry analysis with (open green) and without (shaded, grey) anti-CD31-FITC, anti-UEA-1-FITC, anti-CXCR4-PercP, anti-CD146-FITC, anti-CD29-FITC, anti-CD105-APC, anti-VEGFR2-PE, anti-VE-cadherin-PE, anti-CD63-PE, anti-CD45-PercP and anti-CD133-APC.

5.2.1.2 Immunophenotype characterisation of human endothelial hybridoma line EA.hy926.

Giemsa staining showed these cells were large and granular (Figure 5.2). This was confirmed by flow cytometry analysis of EA.hy926 cells, which showed that the majority of these cells presented high forward and side scatter (FSC/SSC) which is characteristic of large granular cells. Viable cells (gated on FCS/SSC characteristics) showed no expression of CD45, CD133 and CXCR4 surface markers. Around 30% of the EA.hy926 cells expressed CD34; 12.6% were positive for VEGFR2; and 28.83% expressed VE-cadherin. Moreover, most of the cells strongly expressed CD29 (86%), CD31 (88%), UEA-1 (81.77%) and CD146 (98.11%) markers (Figure 5.3). As for the HUVECs, although these results are expressed as percentages of the population not overlapping the unstained negative control, the peaks were in most cases single and generally homogeneous and appear to represent general expression (or lack of expression) by all cells rather than expression only by a subpopulation of cells. However, unlike the HUVECs, clearly for CD34 and to some extent for VE-cadherin there is the appearance of dual peaks suggesting that some major subpopulation of these EA.hy926 cells express these markers more strongly than the remainder, and it may be that these markers reflect the status of these cells in the cell growth cycle. The expression of VEGFR2, VE-cadherin, UEA-1 and CD31 markers on EA.hy926 cells was also demonstrated by immunostaining (Figure 5.4).

a) b)

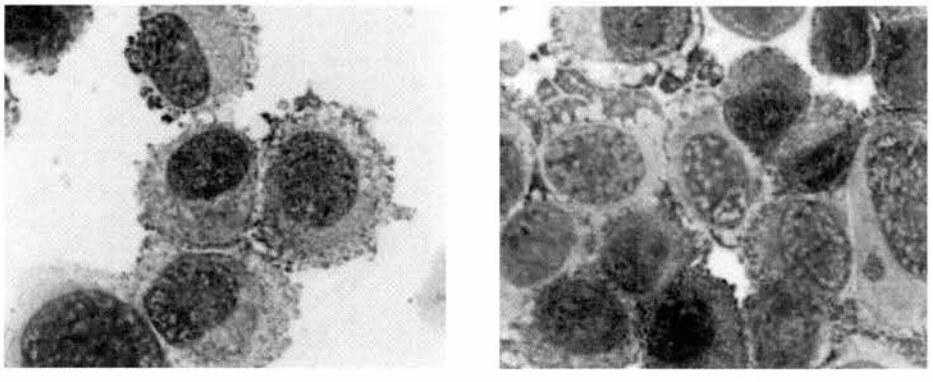


Figure 5.2. Morphology of the EA-hy926 hybridoma endothelial cell line

Harvested cells were used to prepare cytospin slides for Giemsa staining. (a,b) Hybridoma endothelial cells were very large and highly granulated.

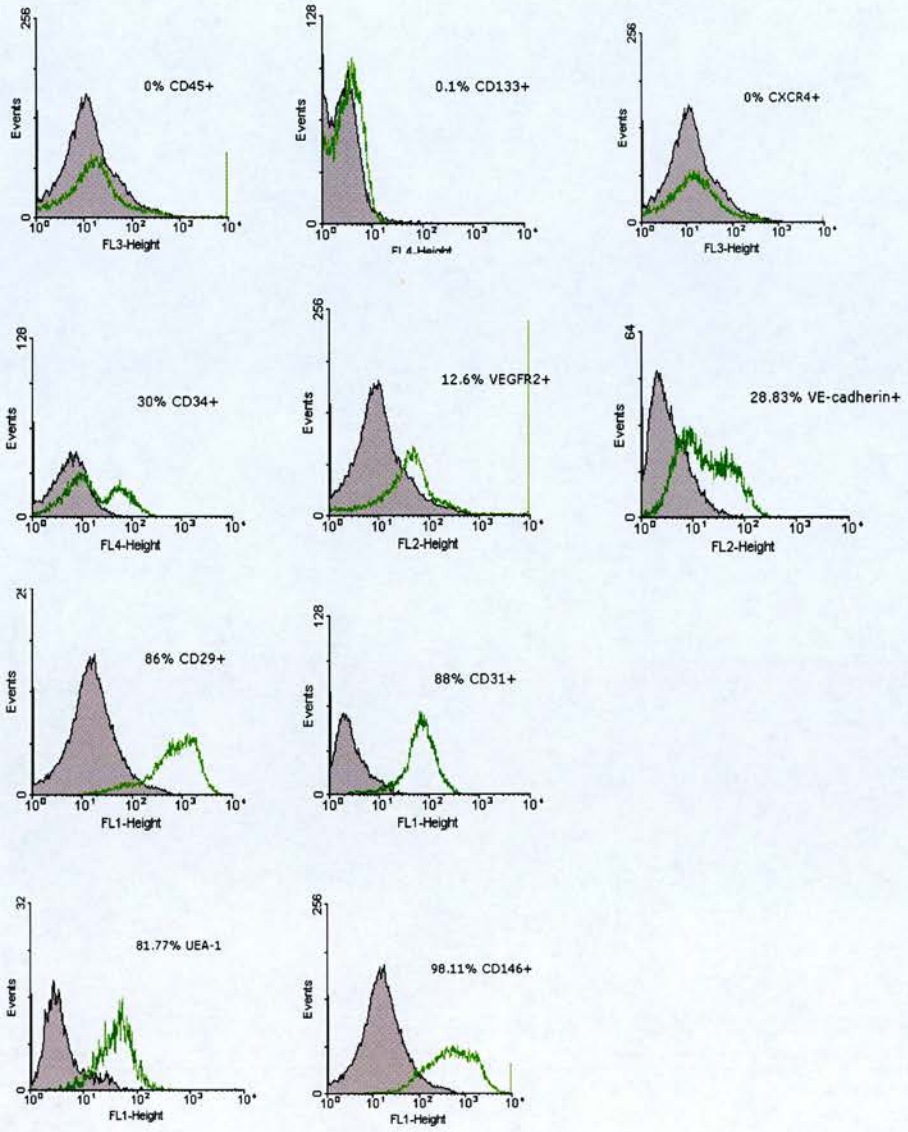


Figure 5.3 Flow cytometry analysis of the EA-hy926 hybridoma endothelial cell line

Flow cytometry analysis with (open green) and without (shaded, grey) anti-CD31-FITC, anti-UEA-1-FITC, anti-CXCR4-PercP, anti-CD146-FITC, anti-CD29-FITC, anti-CD34-APC, anti-VEGFR2-PE, anti-VE-cadherin-PE, anti-CD45-PercP and anti-CD133-APC.

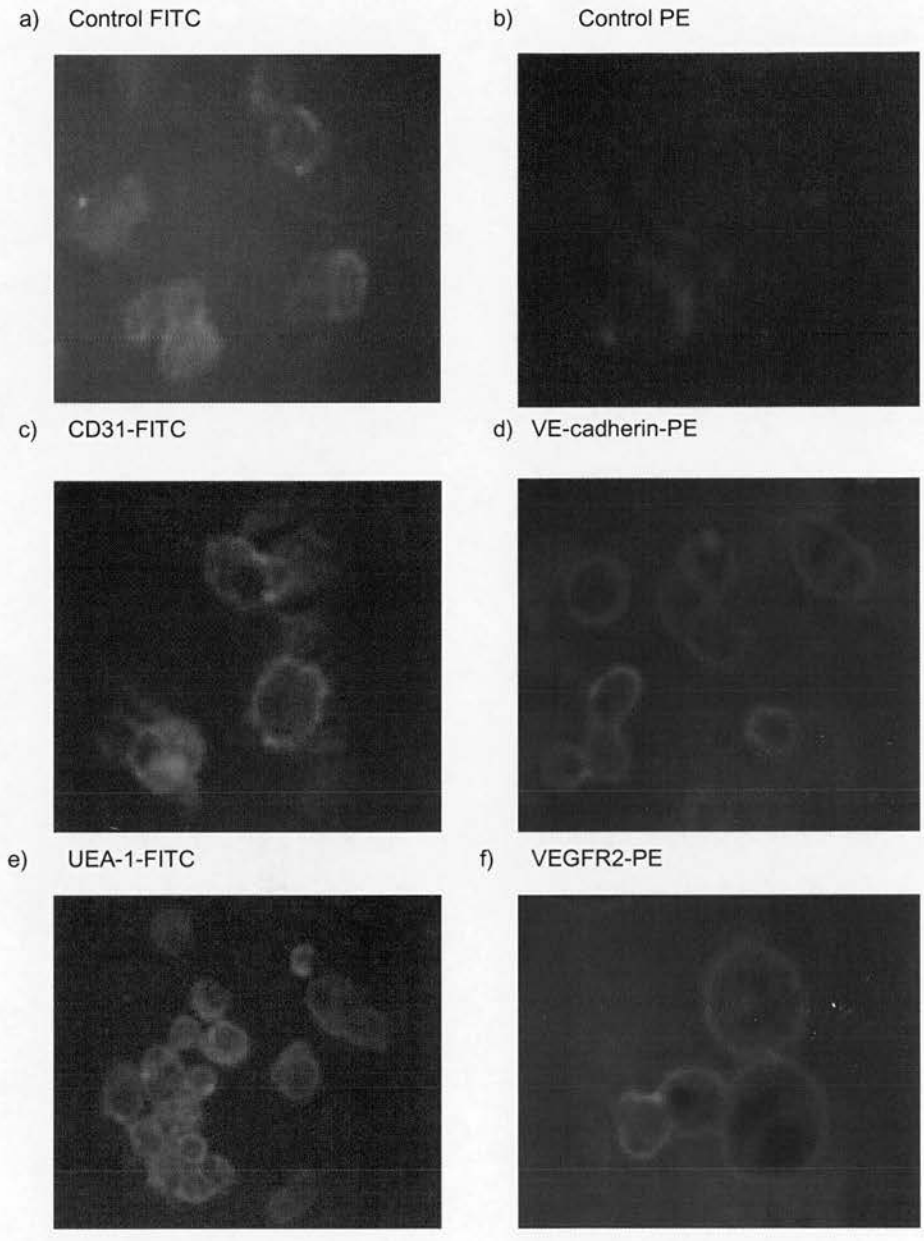


Figure 5.4 Immunofluorescence microscopy of the EA-hy926 hybridoma endothelial cell line. Immunostaining illustrates that the hybridoma endothelial cell line EA-hy926 expressed mature endothelial phenotype markers. a) Cells with no staining pictured under the FITC filter or b) under PE filter. (c-f) cells stained with c) anti-CD31-FITC antibody, d) anti-VE-cadherin-PE, e) *ulex europaeus* lectin agglutinin 1, f) anti-VEGFR2-PE antibody.

5.2.1.3 Functional characterisation of human EC hybridoma line EA.hy926

EA.hy926 cells were also shown to take up DiI-ac-LDL by both flow cytometry (97%) and immunofluoresce staining (Figure 5.5).

EA.hy926 cells were able to form obvious tubular structures following one-week culture in Matrigel (Figure 5.6).

Neither EA.hy926 or HUVEC produced CFU-EPCs in the Hill assay demonstrating that these cells are mature endothelial cells (Figure 5.7).

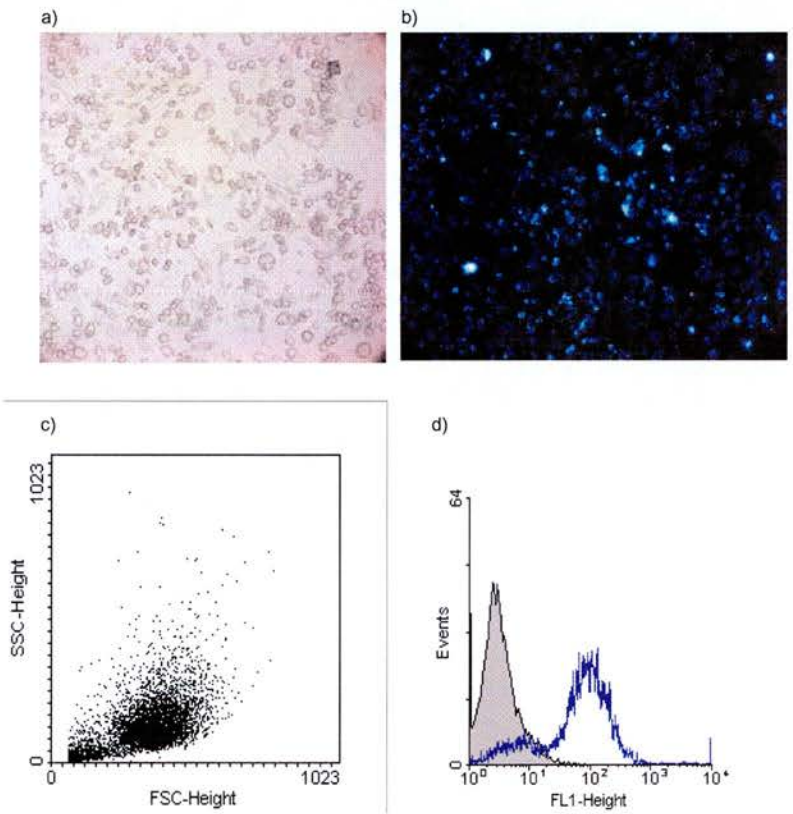


Figure 5.5 DiI-ac-LDL uptake by EA-hy926 hybridoma endothelial cell line.

Ea-hy926 cells took up DiI-ac-LDL like a mature endothelial cell a) negative control and b) with DiI-ac-LDL. c) cells gated in SSC/FCS and d) histogram with (open blue) and without (shaded grey) DiI-ac-LDL staining (FL1-H).

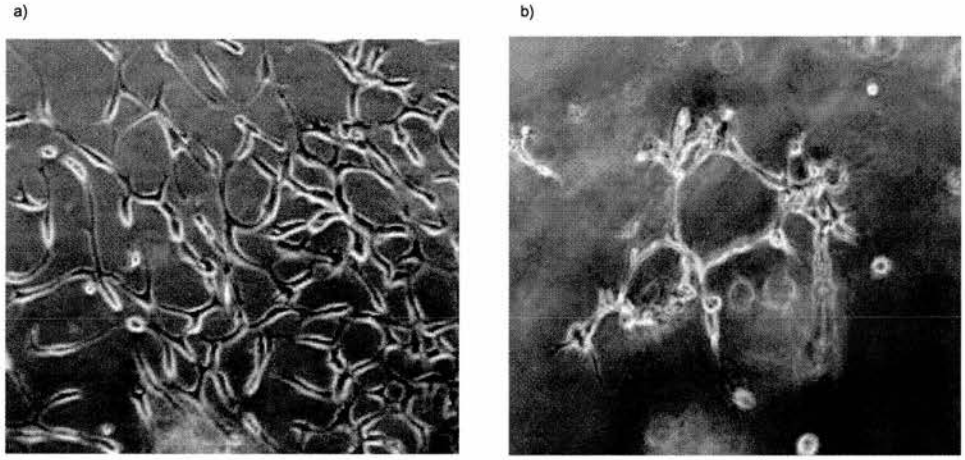


Figure 5.6 Matrigel tubule formation assay by EA-hy926 hybridoma endothelial cell line.

The hybridoma endothelial cells are able to generate capillary-like structures in Matrigel: a) X40 field and b) X100 field

Figure 5.7 Endothelial progenitor cell colony formation (CFU-EPC) by EA-hy926 hybridoma endothelial cell line.

No characteristic colonies are seen when EA-hy926 cells are cultured in CEEM culture.

5.2.1 (b) Peripheral blood sampling

5.2.1.4 Effect of anticoagulants and washing on platelet adherence to peripheral blood mononuclear cells.

(Data obtained in collaboration with Ida Flisijn; visiting student from Noordelijke Hogeschool, Van Hall Instituut, The Netherlands).

Some markers, such as vWf, are expressed on mature endothelial cells and also on platelets. We have found by flow cytometry an apparent high frequency of expression of vWF in different leucocyte subpopulation clusters.The lack of observation of any other antibody or any isotype control binding except CD41 strongly suggests that the binding of vWf, which like CD41 is expressed on platelets, was due to platelet-leucocyte aggregration and not due to any form of non-specific binding.

Different anticoagulants have different effects on platelet-leucocyte aggregation (PLA). Blood taken in heparin tubes showed a high frequency of platelets aggregated with leucocytes, detected by CD41 expression in flow cytometry (Figure 5.8). Similar behaviour was observed using citrate anticoagulant. In contrast EDTA tubes presented much lower platelet-leucocyte aggregation: here it was still present but at a much reduced frequency. We prioritised the use of EDTA tubes for taking blood samples. However, in some clinics blood sampling tubes with EDTA anticoagulant were not available and instead blood was taken into tubes containing heparin anticoagulant. To further study this, blood was taken into heparin tubes and then washed (as whole blood) in PBS/EDTA or PBS/EDTA with trypsin. This apparently reduced PLA formation down to the level seen if EDTA was used as the anticoagulant. There was some gain in reduction of PLA if trypsin was added, but the addition of trypsin could digest some cell antigens and hamper phenotype analysis.

Lignocain (a local anaesthetic) and chloroquine (an antimalarial drug) are known to reduce PLA (Yun *et al.*, 2002) and (Nosal *et al.*, 2000) respectively. Therefore, blood was taken in heparin and then washed with lignocain and chloroquine alone or in combination with EDTA, and compared to washing in EDTA alone or in EDTA/ trypsin (Figure 5.9). Blood taken into heparin and then washed in EDTA/PBS presented a large reduction in platelet-leucocyte aggregation, which further reduced when EDTA/Trypsin had an even a better reduction in PLA, confirming the results shown in Figure 5.8. Washing with lignocain did not apparently reduce PLA formation in heparinised samples, and the reduction seen when washed with lignocain/EDTA probably comes solely from the EDTA. The apparent reduction in

PLA following washing with chloroquine is actually a reduction in CD41 expression by platelets and probably represents no reduction of PLA formation in heparinised samples: as for lignocaine the further reduction seen when washed with chloroquine/ EDTA probably comes solely from the EDTA. The chloroquine did not affect CD45 expression on the leucocytes (not shown): no other markers were studied.
EDTA/PBS was used as a washing solution whenever heparinised samples were received. Although we achieved a reduction in platelet-leucocyte aggregation by choosing to collect blood whenever possible in EDTA tubes, or using EDTA/PBS as a washing solution for blood taken in heparin, PLA formation was not completely abolished. Although vWf is expressed on mature endothelial cells, it is not specific for EC and is also expressed on platelets. Therefore we could not ensure that vWf positive cells seen in flow cytometry in sample analyses were endothelial cells and not more common circulating leucocytes decorated with platelets in PLAs. Therefore vWf was not used as an endothelial marker in our studies.

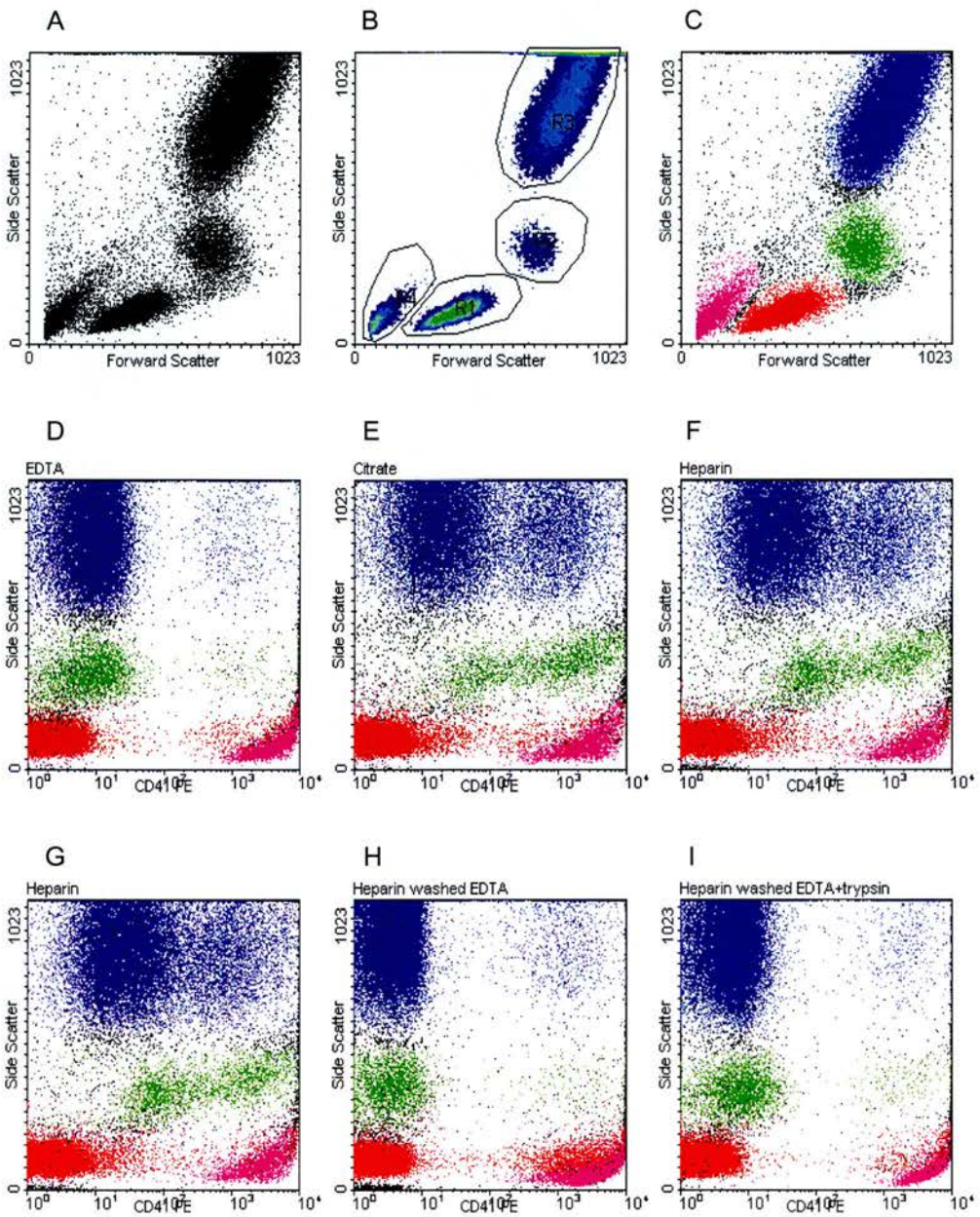

A
B
C
D
EDTA
E
Citrate
F
Heparin
G
Heparin
H
Heparin washed EDTA
I
Heparin washed EDTA+trypsin
Side Scatter
Forward Scatter
CD41PE
R1
R3
0
1023
10^0
10^1
10^2
10^3
10^4

Figure 5.8 Platelet-Leukocyte Aggregation (PLA) in whole blood flow cytometry analysis. Effect of different anticoagulants.

Samples analysed in flow cytometry by "lyse-wash" following immunostaining in whole blood. A. Typical forward-scatter v side-scatter dot-plot showing discrete cell clusters. B. The same cells shown as a density plot to set regions on the major cell concentrations which are (approximately): R1 lymphocytes, R2 monocytes, R3 granulocytes, R4 free platelets. C. The effect of "back-gating" these regions by colour on the forward-scatter v side-scatter dot-plot showing lymphocytes in red, monocytes in green, granulocytes in blue, and free platelets in magenta.

D: Blood taken in EDTA. E: blood taken in Citrate. F: blood taken in Heparin. The pattern of staining with the anti-platelet antibody CD41 shows that large numbers of other leukocytes, especially monocytes and granulocytes, are decorated with adherent platelets when heparin or citrate is used as anticoagulant. The effect is much less when EDTA is used as an anticoagulant, but is not abolished completely.

G: blood taken in Heparin. H: blood taken in Heparin and washed (as whole blood) in EDTA/PBS. I: blood taken in Heparin and washed (as whole blood) in EDTA/PBS with trypsin. Washing heparinised blood samples in the presence of EDTA reduces PLA formation substantially (H). Washing heparinised blood samples in the presence of EDTA and trypsin reduces PLA formation further, but does not abolish it (I).

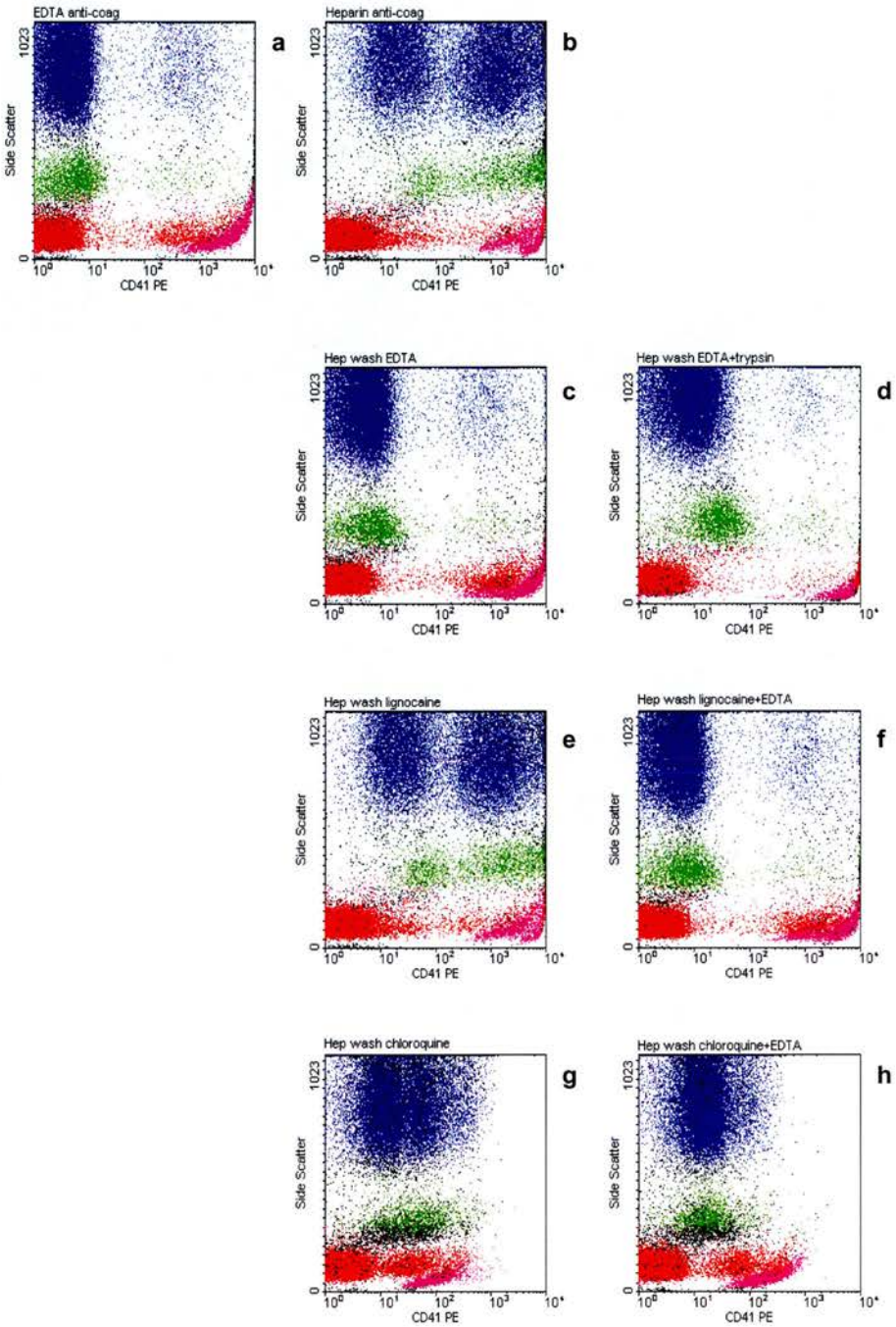

EDTA anti-coag
a
Heparin anti-coag
b
Hep wash EDTA
c
Hep wash EDTA+trypsin
d
Hep wash lignocaine
e
Hep wash lignocaine+EDTA
f
Hep wash chloroquine
g
Hep wash chloroquine+EDTA
h
Side Scatter
1023
0
CD41 PE
10^0 10^1 10^2 10^3 10^4

Figure 5.9 Platelet-Leukocyte Aggregation (PLA) in heparinised whole blood. Effect of different washing protocols.

Samples analysed in flow cytometry by "lyse-wash" following immunostaining in whole blood. Regions were set in a density plot on the major discrete cell foci which are R1 lymphocytes (red), R2 monocytes (green), R3 granulocytes (blue), R4 free platelets (magenta) as for Figure 5.8, using a different sample donor.

Blood taken in EDTA (a) shows much less PLA formation (seen as CD41 staining on different leucocyte populations) compared to blood taken in heparin (b). Blood taken in heparin and washed with (c) EDTA/PBS shows reduction but not abolition of PLA formation, which further reduces if washed with EDTA/PBS containing trypsin (d). Blood taken in heparin and washed with lignocaine/ PBS (e) shows no reduction of PLA formation, but PLA formation reduces if washed with lignocaine/ PBS containing trypsin (f). Blood taken in heparin and washed with chloroquine/PBS (g) shows reduction of CD41 staining on leukocytes, but this appears to come from a reduction of CD41 expression on platelets and not a reduction of PLA formation, and there is some apparent further reduction of CD41 staining if washed with chloroquine /PBS containing trypsin (h).

5.2.1.5 Unexpected expression of VEGFR2

VEGFR2 is thought to be specifically expressed by endothelial precursors and mature cells. However, analysis of whole blood showed that other cells, in particular granulocytes and monocytes, expressed VEGFR2 (Figure 5.10). Though this was not seen in all samples tested. The incidence apparently of aberrant VEGFR2 expression was highest in mobilised peripheral blood patient samples (60%, n=20) whereas it was not detected in mobilised peripheral blood donor samples (0%, n=4), though this latter group was very small. In the non-mobilised sources tested the highest incidence was seen in bone marrow (33%, n=9) and the lowest in normal peripheral blood (6.25%, n=16), whilst in cord blood it was intermediate (30%, n=20) (Table 5.3). Similar results were seen in peripheral blood samples taken from patients participating in a clinical study of angioplasty. VEGFR2 expression was detected in samples taken before, immediately after and at 6h and 24h following procedure. VEGFR2 expression did not appear to be specifically associated with any aspect of the procedure. Some patients were null at all time points, other were positive at all time points whereas some appeared to up-regulate expression immediately following the procedure (Figure 5.11).

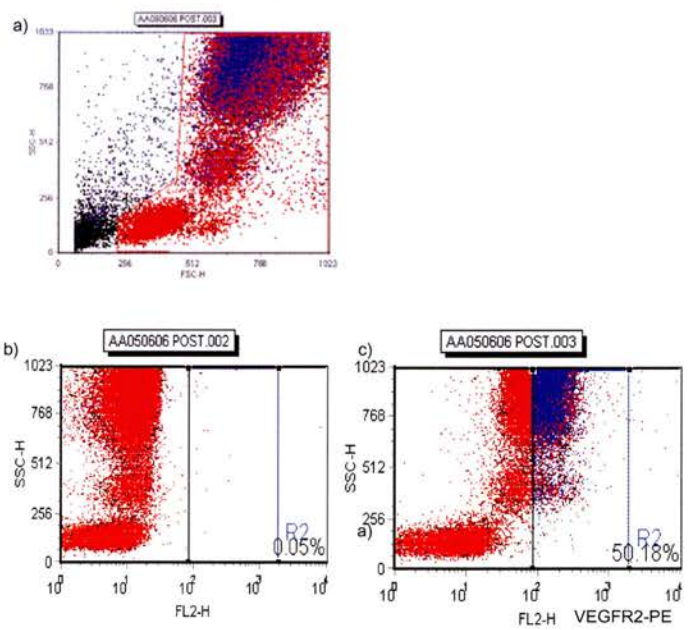


Figure 5.10. VEGFR2 unexpected binding

Whole blood flow cytometry analysis of the expression of VEGFR2-PE (R&D systems), a) FCS/SSC, b and c) without and with anti-VEGFR2-PE antibody (blue) gated in FCS/SSC. VEGFR2 unexpected expression in the area of granulocytes and monocytes. Blood source: G-CSF mobilised peripheral blood.

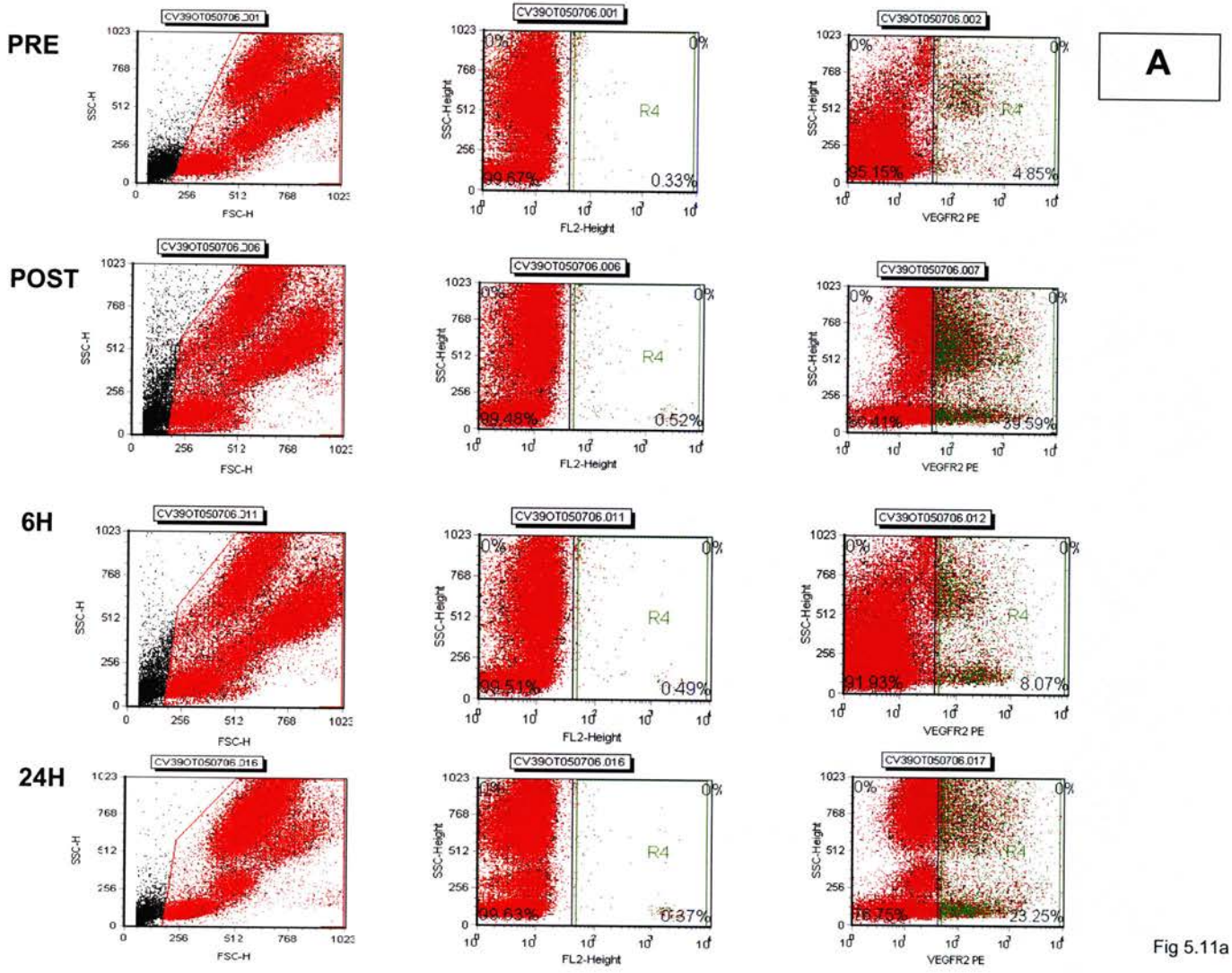

PRE
POST
6H
24H
A
CV39OT050706.001
CV39OT050706.002
CV39OT050706.006
CV39OT050706.007
CV39OT050706.011
CV39OT050706.012
CV39OT050706.016
CV39OT050706.017
SSC-H
FSC-H
SSC-Height
FL2-Height
VEGFR2 PE
R4
0%
0.33%
4.85%
0.52%
39.59%
0.49%
8.07%
0.37%
23.25%

Fig 5.11a

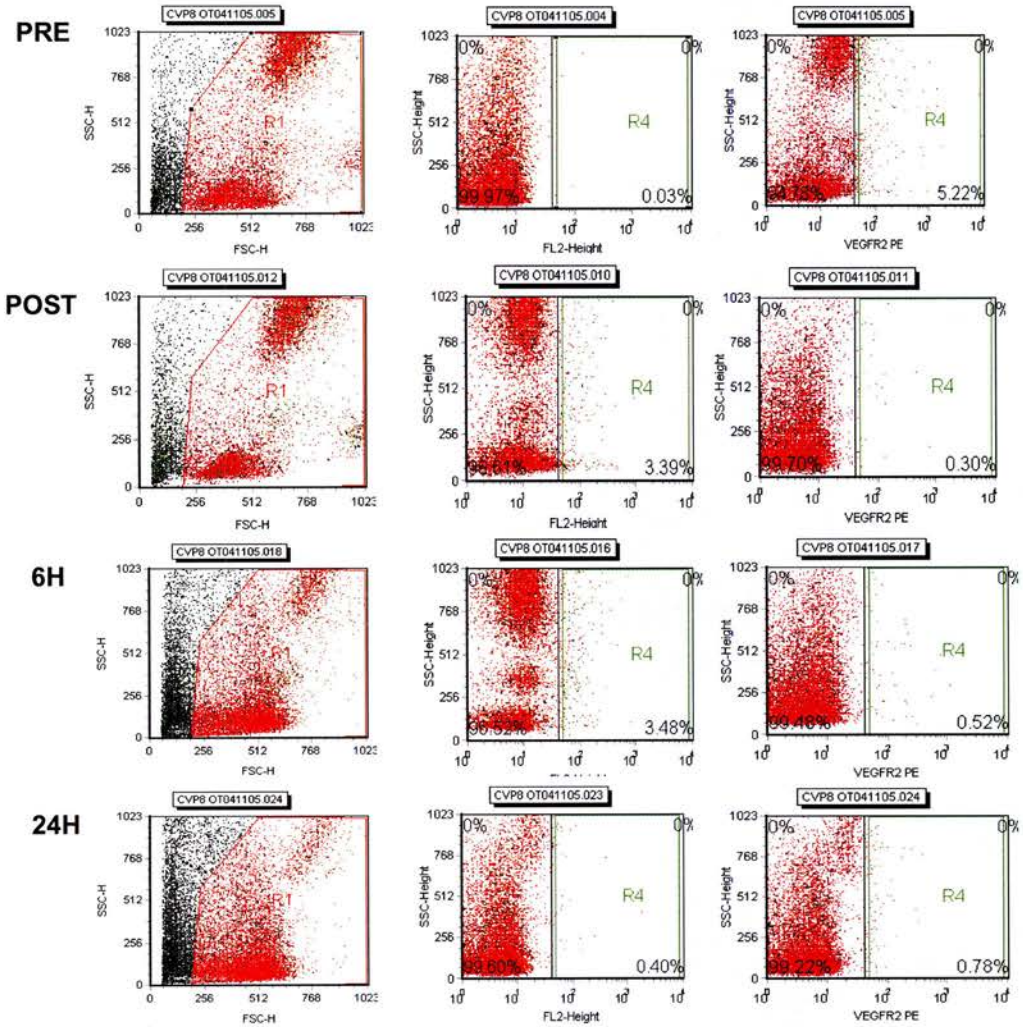
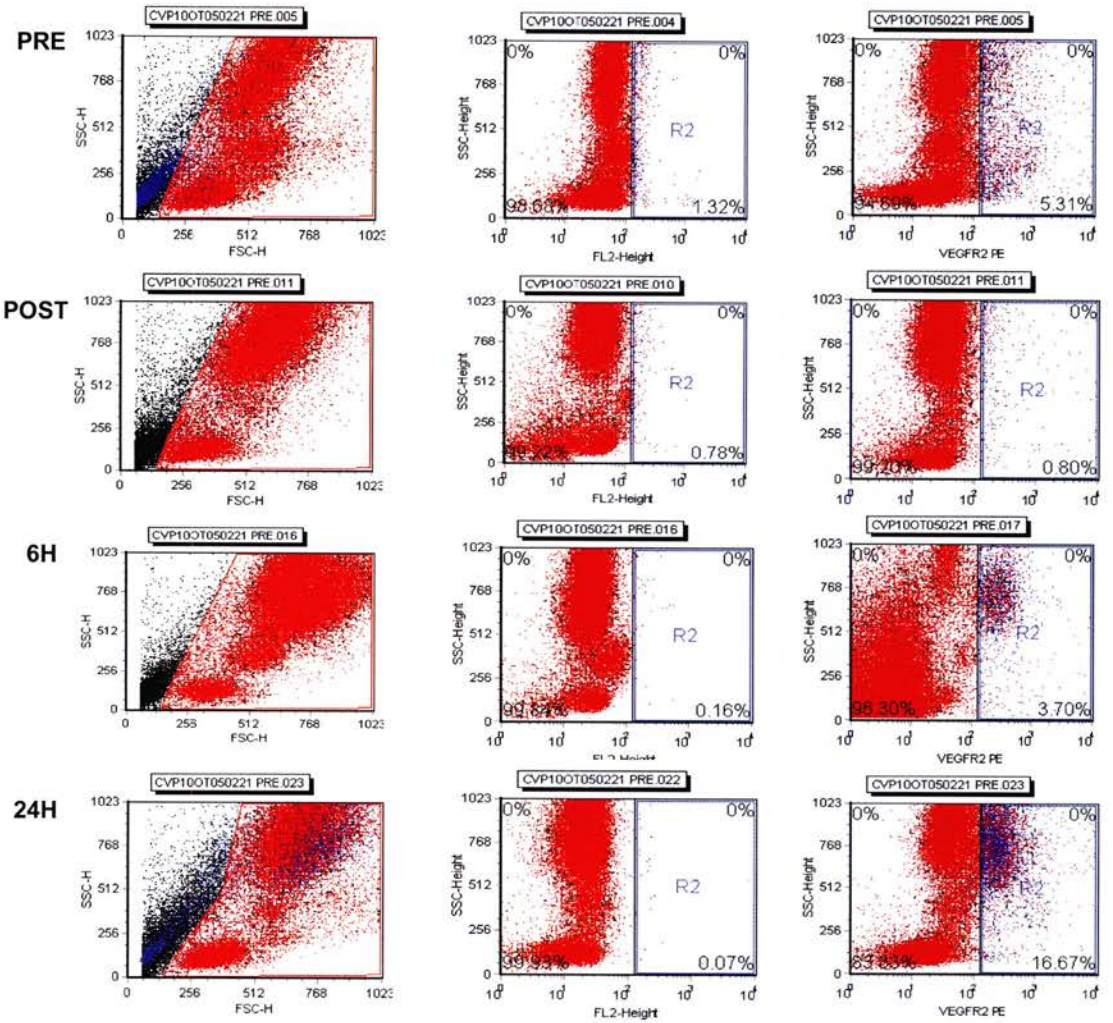

PRE
POST
6H
24H
B
CVP8 OT041105.005
CVP8 OT041105.004
CVP8 OT041105.012
CVP8 OT041105.010
CVP8 OT041105.011
CVP8 OT041105.018
CVP8 OT041105.016
CVP8 OT041105.017
CVP8 OT041105.024
CVP8 OT041105.023
SSC-H
FSC-H
SSC-Height
FL2-Height
VEGFR2 PE
R1
R4
0.03%
5.22%
3.39%
0.30%
3.48%
0.52%
0.40%
0.78%

Fig 5.11b


PRE
POST
6H
24H
CVP10OT050221 PRE.005
CVP10OT050221 PRE.004
CVP10OT050221 PRE.005
CVP10OT050221 PRE.011
CVP10OT050221 PRE.010
CVP10OT050221 PRE.011
CVP10OT050221 PRE.016
CVP10OT050221 PRE.016
CVP10OT050221 PRE.017
CVP10OT050221 PRE.023
CVP10OT050221 PRE.022
CVP10OT050221 PRE.023
SSC-H
SSC-Height
FSC-H
FL2-Height
VEGFR2 PE
R2
0%
1.32%
5.31%
0.78%
0.80%
0.16%
3.70%
0.07%
16.67%

C

Fig 5.11c

Figure 5.11 VEGFR2 unexpected binding: different expression patterns

Figures 5.11, A,B and C show whole blood flow cytometry expression of VEGFR2-PE (R&D systems) in different sample patient samples. A) VEGFR2 expression was detected in samples taken before, immediately after and at 6h and 24h following procedure, B) VEGFR2 expression was not seen in any of the samples time points and C) VEGFR2 expression changed depending on the time point of the clinical procedure. Blood source: patient samples undergoing percutaneous coronary intervention (PCI).

	mPBP (n=20)	mPBD (n=4)	nPB (n=16)	BM (n=9)	CB (n=20)
VEGFR2	60%	0%	6.25%	33%	30%
VE-cadherin	90%	75%	93.75%	100%	100%

Table 5.3 VEGFR2 and VE-cadherin unexpected binding: percentage of the cases

Proportion of the samples with VEGFR2 and VE-cadherin unexpected expression in the different sources analysed (non-mobilised) peripheral blood (nPB) from healthy volunteers, bone marrow (BM), umbilical cord blood (CB) and G-CSF-mobilised peripheral blood samples (mPBP, patients for autologous grafts; or mPBD, donors for allogeneic grafts).

5.2.1.6 Unexpected expression of VE-cadherin

VE-cadherin has also been proposed as a marker of mature endothelial cells. Endothelial cell populations in all sources were found to express VE-cadherin. However, non-endothelial cell populations were also found to express VE-cadherin. This was more frequent than the above expression of VEGFR2, and seemed to be associated with different lineages. 90% of mobilised peripheral blood patient samples (n=20) and 75% of mobilised peripheral blood donor (n=4) samples were positive, whereas non-mobilised samples showed an even higher incidence, 100% of bone marrow (n=9), 100% of cord blood (n=20) and 93.75% of normal peripheral blood samples (n=16) (Figure 5.12) (Table 5.3).

VE-cadherin expression in mobilised peripheral blood samples was associated with cells having scatter characteristics of monocytes and lymphocytes, whilst that in cord blood appeared to be limited to lymphocytes, specifically $CD8^+$T cells (Figure 5.13).

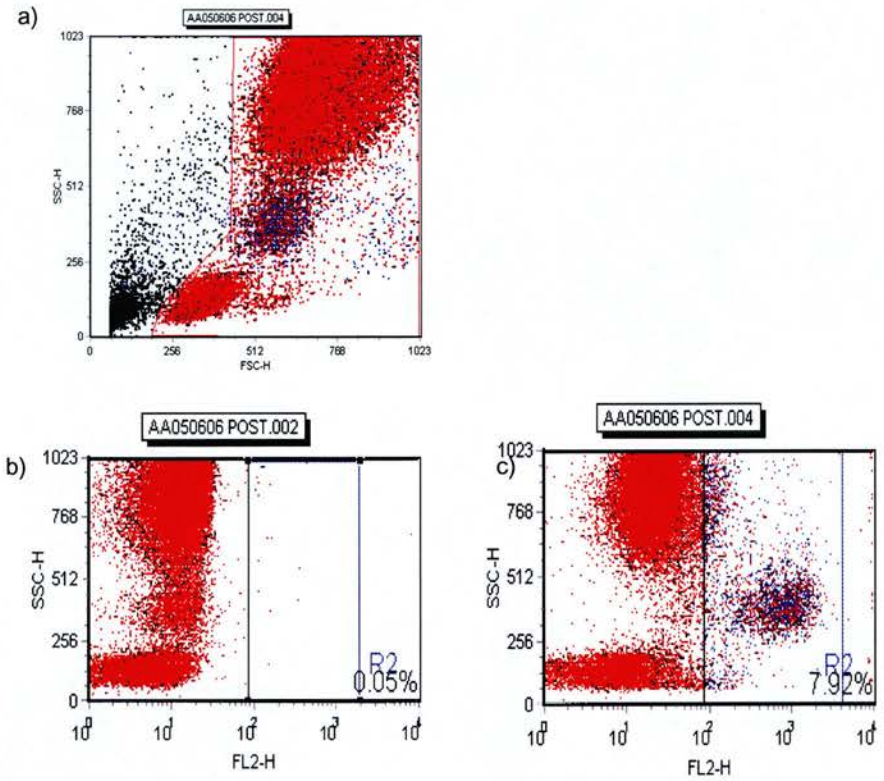


Figure 5.12 VE-cadherin unexpected binding

Whole blood flow cytometry analysis of the expression of VE-cadherin-PE (Santa Cruz Biotechnology), a) FCS/SSC, b and c) without and with anti-VE-cadherin-PE antibody (blue) gated in FCS/SSC. VE-cadherin unexpected expression mostly in the area of monocytes. Blood source: G-CSF mobilised peripheral blood.

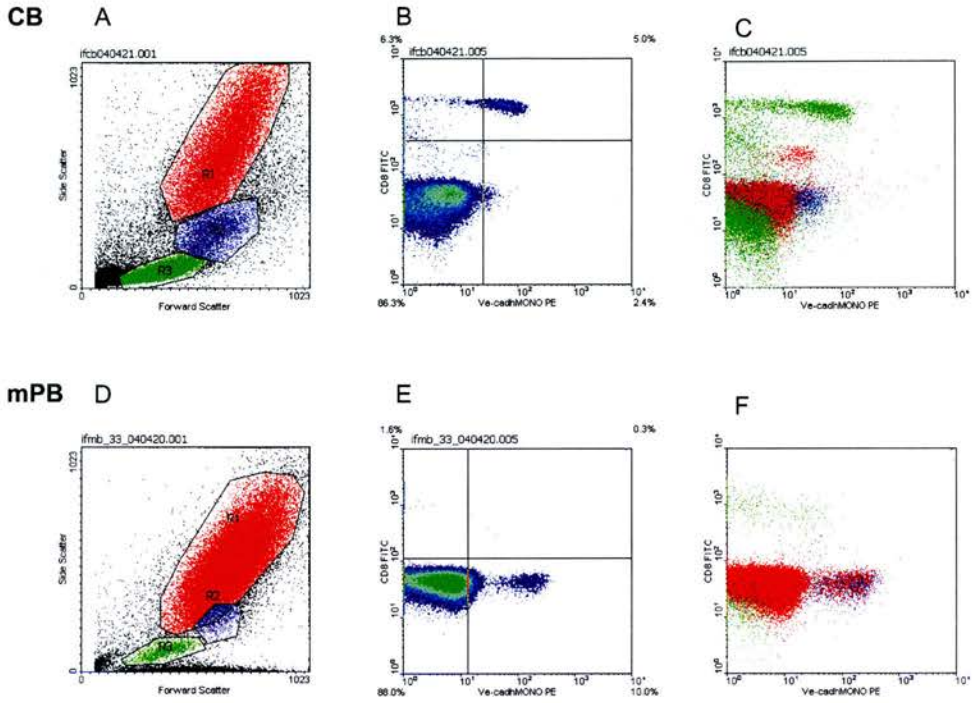


Figure 5.13 VE-cadherin unexpected binding: Cord blood compared to G-CSF mobilised sources.

(A) Cord blood, FSC/SSC, (B) Density plot (C) and Dot plot VE-cadherin against CD8. (D) G-CSF mobilised blood, FSC/SSC, (E)Density plot and (F) Dot plot VE-cadherin against CD8.Regions: Granulocytes (red), Monocytes (blue), and Lymphocytes (green).

5.2.2 Analysis of markers associated with endothelial progenitor phenotypes in sources of haematopoietic stem cells and in normal peripheral blood.

5.2.2.1 Numbers of leucocytes in samples

Total white blood cells per ml (wbc/ml) in the various samples were counted in a haemocytometer using white cell diluting fluid. No significant differences were detected between the various sources used.

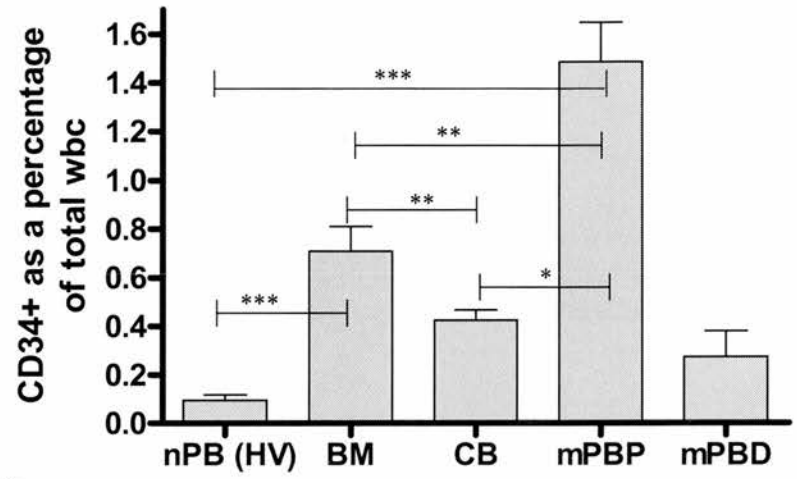
5.2.2.2 Quantification of $CD34^+$ cells.

The expression of CD34 on leucocytes was analysed in 10 bone marrow (BM); 21 cord blood (CB); and 32 mobilised peripheral blood (mPB), of these 27 were patients for autologous transplant (mPBP) and 5 were donors for allogeneic transplantation (mPBD). The results showed that as a fraction of total wbc the proportion of $CD34^+$ cells was highest in mPBP samples at 1.46% as compared with bone marrow samples at 0.71% and CB samples at 0.42% and that many of these differences in percentages were significant (Figure 5.14a and Table 5.4). When total numbers of $CD34^+$ cells are calculated from wbc/ml values, both mPBP and BM had significantly higher numbers of $CD34^+$ than cord blood (Table 5.5). Normal peripheral blood (not mobilised) (nPB) (n=16) presented a very low percentage and number of $CD34^+$ cells (0.09%) compared to the other sources tested (Figure 5.14a; Tables 5.4 and 5.5).

5.2.2.3 Quantification of $CD133^+$ cells.

The expression of CD133 on leucocytes was analysed in 8 bone marrow (BM); 7 cord blood (CB); and 10 mobilised peripheral blood (mPB), of these 7 were patients for autologous transplant (mPBP) and 3 were donors for allogeneic transplantation (mPBD). The distribution of $CD133^+$ cells between sample groups was similar to that of $CD34^+$ cells in that again mPBP had the highest proportion of $CD133^+$ cells (mPBP samples at 1.04% as compared with BM samples at 0.46% and CB samples at 0.41%) (Figure 5.14b and Table 5.4). Total numbers of $CD133^+$ were higher in mPBP and BM compared to CB (Table 5.5). G-CSF administration appears to mobilise $CD34^+$ and $CD133^+$ cells in similar ways. Similar to $CD34^+$ cells, the proportion and numbers of $CD133^+$ cells in peripheral blood (nPB) (n=16) were very low compared to the other sources tested (Figure 5.14b; Table 5.4 and 5.5).

a)



b)

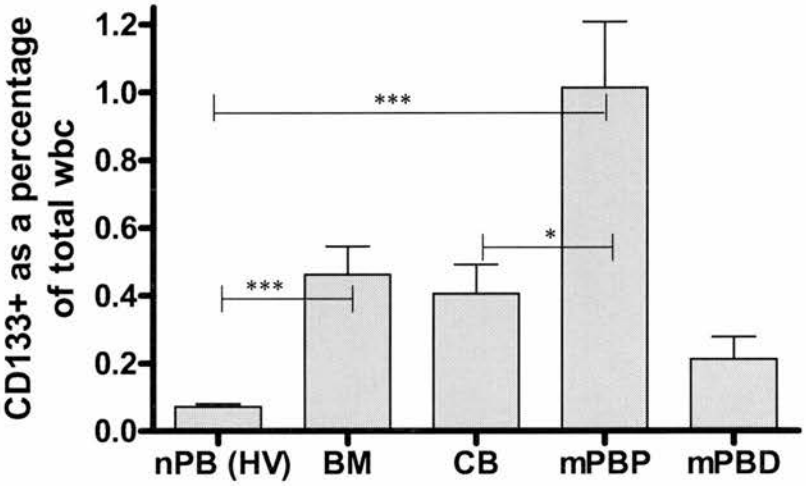


Figure 5.14 Expression of CD34 or CD133 markers in haematopoietic stem cell clinical transplant sources and normal blood.

Percentage (of the total white blood cells) of cells expressing CD34 (a) or CD133 (b) in the different tested sources. Sources were normal (non-mobilised) peripheral blood (nPB) from healthy volunteers (HV), bone marrow (BM), umbilical cord blood (CB) and G-CSF-mobilised peripheral blood samples (mPBP, patients for autologous grafts; or mPBD, donors for allogeneic grafts). (* = $p<0.05$; ** = $p< 0.01$; *** = $p<0.001$, Mann-Whitney test)

5.2.2.4 Quantification of CD133 and CD34 co-expression.

Analysis of CD133 expression by $CD34^+$ cells in each sample group showed that both the mobilised blood (mPB) groups (mPBP and mPBD) (79.76% and 71.78% respectively) had significantly higher co-expression of CD133 by $CD34^+$ cells as compared to CB (53%) or BM (13%). The low co-expression of $CD133^+$ on $CD34^+$ cells in BM was similarly low in normal peripheral blood (nPB) (Figure 5.15 and Table 5.4).

Analysis of the proportion of $CD133^+$ cells which were CD34-negative showed that this population is higher in BM (31.2%), CB (21.7%) and in nPB (33.1%) by comparison with that of mobilised blood, mPBP (9.13%) and mPBD (11.9%) (Figure 5.15 and Table 5.4).

Similarly, the proportion of $CD34^+$ cells which are CD133-negative was higher in BM (63%), CB (35%) and in nPB (62.1%) by comparison with mobilised blood, mPBP (15.85%) and mPBD (21.22%) (Figure 5.15 and Table 5.4).

It was noted that mobilised blood from healthy allogeneic HSC donors showed similar CD34 and CD133 characteristics to mobilised blood from patients donating HSC for autologous transplant, which infers that these characteristics are related to G-CSF administration and HSC mobilisation rather than any underlying condition related to the patients leukaemic diseases (in remission) or their chemotherapy. This was true for all the results in this chapter where these sets of samples were compared.

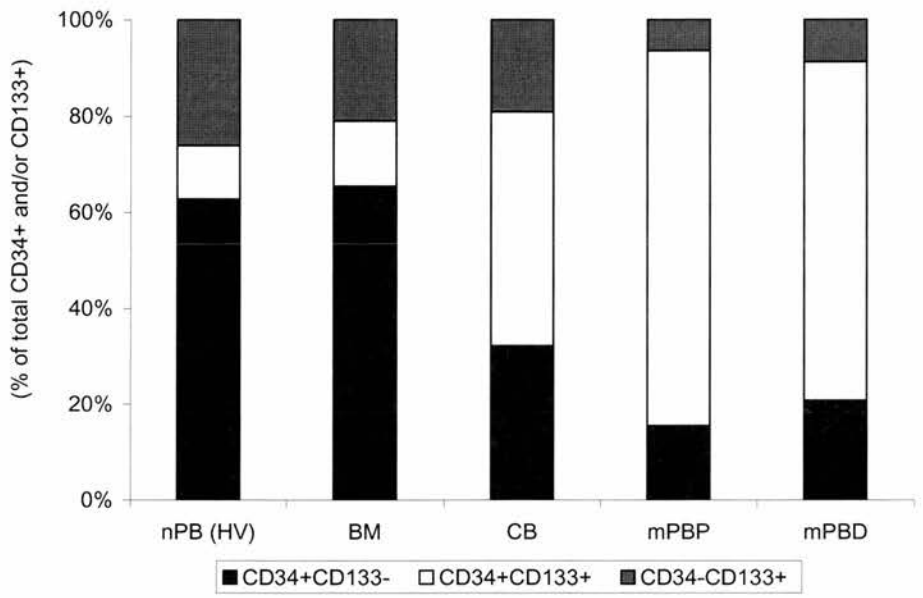


Figure 5.15 Percentage of cells expressing CD34 without CD133, CD133 without CD34, or co-expressing both markers in haematopoietic stem cell clinical transplant sources and normal blood.

Percentages (of their summed populations) of $CD34^+$ cells negative for CD133 expression (black), $CD133^+$ cells negative for CD34 expression (hatched) and cells co-expressing CD133 and CD34 markers (white) were compared between the various sources tested. Normal peripheral (non-mobilised) blood (nPB) from healthy volunteers (HV) (n=16), Bone marrow (BM) (n=8), Cord blood (CB) (n=7) and G-CSF mobilised peripheral blood samples (mPBP, autologous patients (n=7); or mPBD, allogeneic donors (n=3)).

5.2.2.5 Quantification of CD34$^+$ CD45^{dim} cells

CD45 is a recognised pan-leucocyte and haematopoietic cell marker. A preponderance of CD34$^+$ cells express CD45 brightly, however a small number of CD34$^+$ cells are CD45 dim or negative. These CD34$^+$ CD45^{dim} cells are virtually absent in mobilised blood sources; mPBP (1.93%) or mPBD (1.81%).

BM (11.08%), CB (15.08%) or nPB (37.34%) (Figure 5.16 and Table 5.4) showed a significantly higher proportion of CD34$^+$CD45^{dim} than mobilised blood sources. The majority of CD34$^+$ cells in mPB have bright expression of CD45, which may indicate that these cells are already destined to generate haematopoietic cells and their potential to develop along other lineage pathways e.g. endothelial may have been lost.

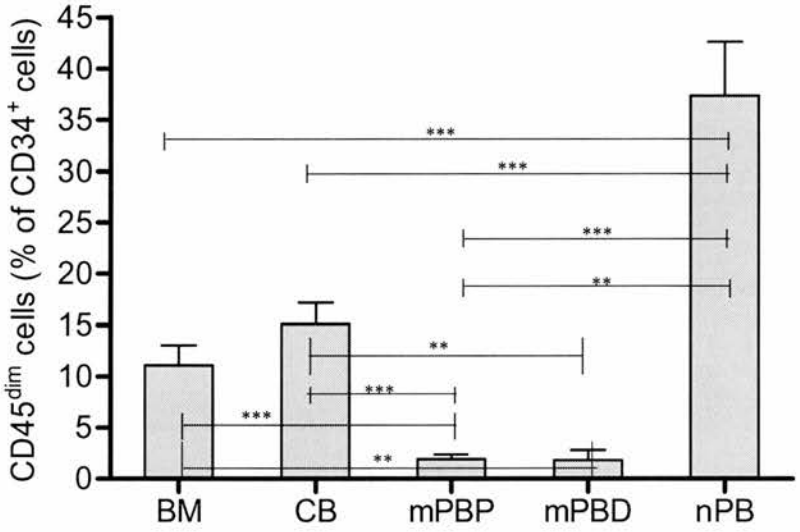


Figure 5.16 Percentage of cells expressing CD34 but weak for CD45 expression in haematopoietic stem cell clinical transplant sources and normal blood

Percentage of CD34$^+$ cells which are weak to negative for CD45 expression in the different sources tested. Normal (non-mobilised) peripheral blood (nPB) (n=16), bone marrow (BM) (n=10), cord blood (CB) (n=21) and G-CSF-mobilised peripheral blood samples (mPBP, patient (n=27); or mPBD, donor (n=5)). (* = $p<0.05$; ** = $p< 0.01$; *** = $p<0.001$, Mann-Whitney test)

5.2.2.6 Quantification of VEGFR2$^+$ cells as a proportion of the CD34$^+$ cells (CD34$^+$VEGFR2$^+$).

In the CD34$^+$-rich HSC sources (i.e. excluding nPB), CB (8.93%) was the source with the highest percentage of CD34$^+$ expressing VEGFR2$^+$ cells, compared to BM (5.43%), mPBP (3.92%) or mPBD (4.19%), (Figure 5.17 and Table 5.4). Although cord blood had the highest percentage of CD34$^+$VEGFR2$^+$ it was the HSC source with the lowest total number of CD34$^+$ cells and therefore showed no significant difference from BM and mobilised peripheral blood samples in terms of numbers of CD34$^+$VEGFR2$^+$ cells (Table 5.5).

The percentage of CD34$^+$ cells co-expressing VEGFR2 was significally higher in normal peripheral blood (24.49%) than in any other source tested, even though normal peripheral blood is relatively poor in CD34$^+$ by comparison (Figure 5.16 and Table 5.4). However, due to the very low number of CD34$^+$ cells in normal peripheral blood, the total number of CD34$^+$VEGFR2$^+$ co-expressing cells was significally lower in normal peripheral blood than in cord blood or BM (Table 5.5).

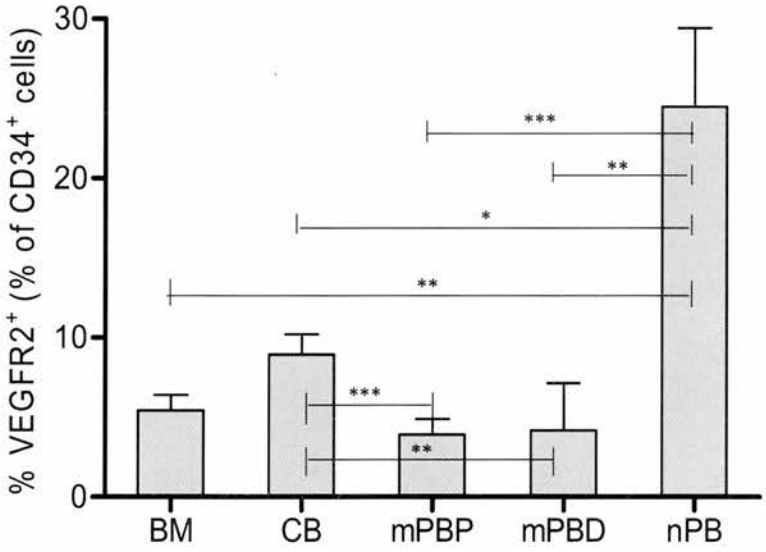


Figure 5.17 Percentage of VEGFR2$^+$ cells as a fraction of the CD34$^+$ cells in haematopoietic stem cell clinical transplant sources and normal blood

Percentage of CD34$^+$ cells co-expressing VEGFR2 in the different sources tested. Normal peripheral blood (nPB) (n=16), bone marrow (BM) (n=10), cord blood (CB) (n=21) and G-CSF-mobilised peripheral blood samples (mPBP, patient (n=27); or mPBD, donor (n=5)). (* = $p<0.05$; ** = $p< 0.01$; *** = $p<0.001$, Mann-Whitney test)

5.2.2.7 Quantification of VEGFR2$^+$ cells as a fraction of the CD133$^+$ cells (CD133$^+$VEGFR2$^+$)

Normal peripheral blood (nPB) was also the source with highest percentage of CD133$^+$VEGFR2$^+$ (23.79%) cells compared to the other sources tested whereas bone marrow had the lowest percentage (0.32%) (Figure 5.18a and Table 5.4). However due to the low number of CD133$^+$ cells in normal peripheral blood the total number of CD133$^+$ VEGFR2$^+$ cells was similar to the numbers obtained in cord blood and lower than mPBP (Table 5.5).

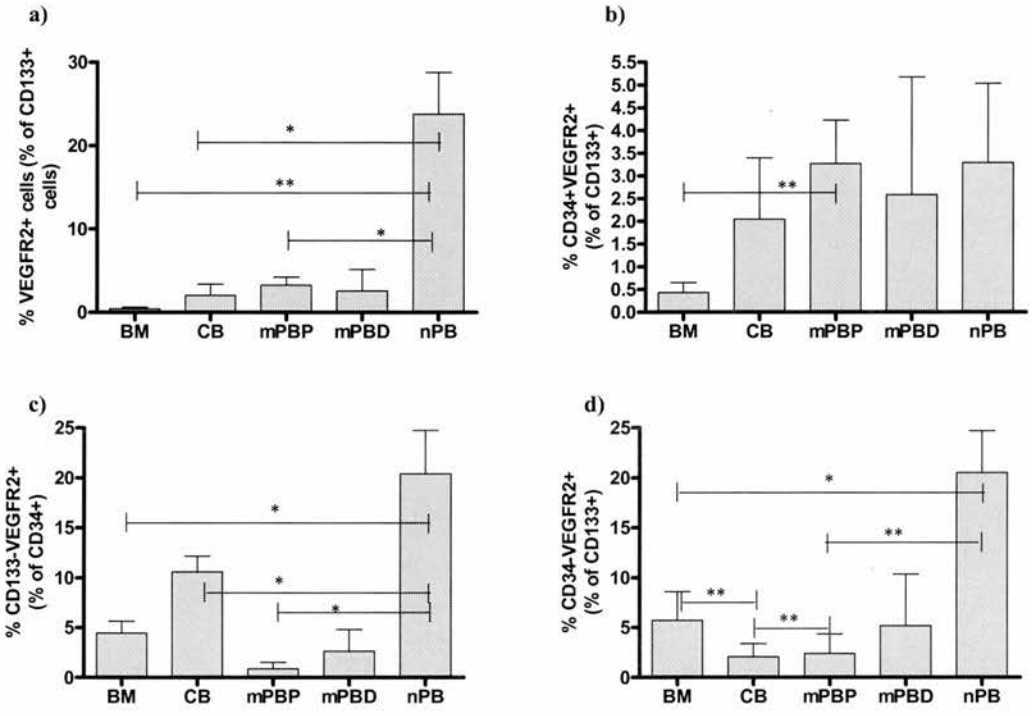


Figure 5.18 Pattern of VEGFR2 expression with CD34 and/or CD133 expression in haematopoietic stem cell clinical transplant sources and normal blood

(a) Percentage of CD133$^+$ cells co-expressing VEGFR2, (b) Percentage of CD133$^+$ cells co-expressing CD34 and VEGFR2 (c) Percentage of CD34$^+$ cells expressing VEGFR2 without expressing CD133, (d) Percentage of CD133$^+$ expressing VEGFR2 without expressing CD34, in the different sources tested. Normal peripheral blood (nPB)(n=16), bone marrow (BM)(n=8), cord blood (CB) (n=7) and G-CSF-mobilised peripheral blood samples (mPBP, patient (n=7); or mPBD, donor (n=3)). (* = $p<0.05$; ** = $p< 0.01$; *** = $p<0.001$, Mann-Whitney test)

5.2.2.8 Quantification of $CD133^-$ $VEGFR2^+$ cells as a fraction of the $CD34^+$ cells ($CD34^+CD133^-VEGFR2^+$).

The highest percentage of $CD34^+$ cells co-expressing VEGFR2 but not CD133 was seen in cord blood (10.59%) and normal peripheral blood (20.42%) whereas the percentage in BM and mPBP was much lower at (4.47%) and (0.89%) respectively. Therefore, the majority of $CD34^+VEGFR2^+$ cells in cord blood and normal peripheral blood were CD133-negative (Figure 5.18c and Table 5.4).

5.2.2.9 Quantification of $CD34^+VEGFR2^+$ cells as a fraction of the $CD133^+$ cells ($CD133^+CD34^+VEGFR2^+$).

The $CD133^+CD34^+VEGFR2^+$ population, positive for all three markers, was almost below limits of detection by flow cytometry when acquisition of a minimum of 50,000 leucocyte events was used. The sources with higher proportions of $CD34^+CD133^+VEGFR2^+$ were mPB (3.27% mPBP; 2.59% mPBD) and nPB (3.29%) as compared to BM (0.43%) and CB (2.05%) (Figure 5.18b and Table 5.4).

5.2.2.10 Quantification of $CD34^-VEGFR2^+$ cells as a fraction of $CD133^+$ cells ($CD133^+CD34^-VEGFR2^+$).

This population did not show significant differences in terms of percentages between the different sources analysed with the exception of peripheral blood which was significantly higher than the other sources tested (Figure 5.18d and Table 5.4).

5.2.2.11 Mature circulating endothelial cells (CEC).

Circulating endothelial cells (CEC) are extremely rare in peripheral blood. To demonstrate the presence of these cells in the sources studied, the frequency of cells expressing CD146 but negative for expression of CD3 and CD45 were assessed ($CD146^+CD3^-CD45^-$). As shown in Figure 5.19 and Table 5.4 these cells were highest in bone marrow followed by cord blood, and lower in mobilised peripheral blood and normal peripheral blood.

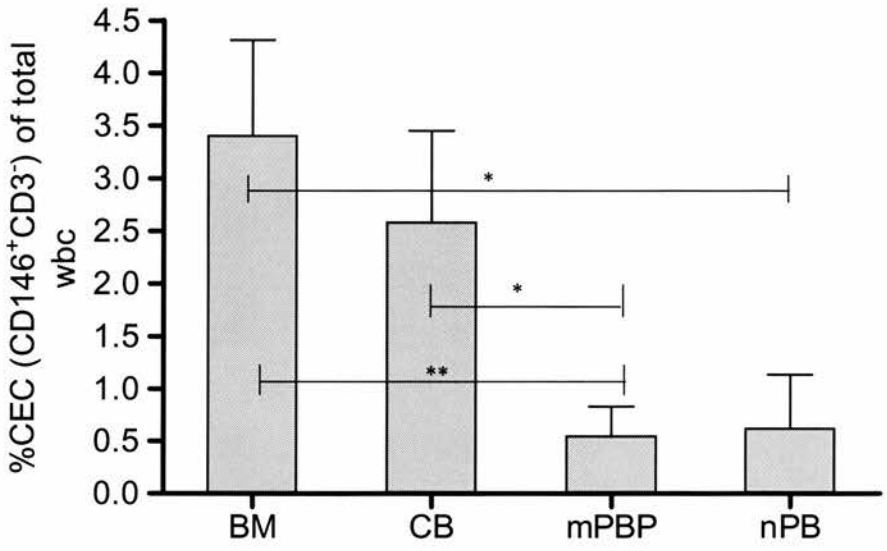


Figure 5.19 Percentage of mature circulating endothelial cells in haematopoietic stem cell clinical transplant sources and normal blood

Percentage of cells expressing CD146 but negative for expression of CD3 and CD45 markers ($CD146^+CD3^-CD45^-$). Normal peripheral blood (nPB)(n=6), bone marrow (BM)(n=6), cord blood (CB) (n=6) and G-CSF-mobilised peripheral blood samples (mPBP, patients)(n=8) (* = $p<0.05$; ** = $p< 0.01$; *** = $p<0.001$, Mann-Whitney test)

5.2.3 Analysis of endothelial progenitor cells in sources of haematopoietic stem cells and in normal peripheral blood using a colony assay (CFU-EPC).

5.2.3 (a) Endothelial progenitor cell colony forming unit (CFU-EPC) frequencies in sources of haematopoietic stem cells and in normal peripheral blood

The endothelial colony assay described by Hill *et al.*, (2003) was used to assess the frequency of CFU-EPC for each of the potential sources of EPC studied. Hill *et al.*, (2003) used a 48h primary fibronectin adhesion step to deplete mature EC and phagocytes. CFU-EPC develop from the non-adherent cells on secondary culture on fibronectin for a further 3 days. CFU-EPC are described as a core of round cells surrounded by spindle-shaped cells *(see Chapter 2 section 2.4.1.2).*

Of the sources shown to be rich in $CD34^+$ cells (i.e. BM, CB and mPBP), bone marrow gave the highest frequency of CFU-EPC. Of these HSC-rich sources, bone marrow was by far the best source, cord blood was lower, and G-CSF mobilised peripheral blood (mPB) was virtually incapable of CFU-EPC generation, again similar for both autologous patients (mPBP) or allogeneic donors (mPBD) (BM: 15.7 CFU-EPC/10^6 cells plated; CB: 6.4 CFU-EPC/10^6 cells plated; mPBP: 0.9 CFU-EPC/10^6 cells plated; mPBD: 0.6 CFU-EPC/10^6 cells plated) (Figure 5.20 and Table 5.4).

However, the source which gave much the highest frequency of CFU-EPC was normal peripheral blood, where the numbers of $CD34^+$ and $CD133^+$ cells are very low. Following the primary depletion step peripheral blood MNCs generated an average of 39.37 CFU-EPC/10^6 cells plated; thus 1 in 25,400 cells had the potential to proliferate and generate endothelial colonies. After further time in culture, CFU-EPC dissociated and the round cells from the core of the colony died or became spindle shaped cells. By day 14 all cells were spindle-shaped, did not appear to proliferate, but had elongated and interacted. These cells stained positive for DiI-Ac-LDL and UEA-1 markers and remained alive for up to 2 months.

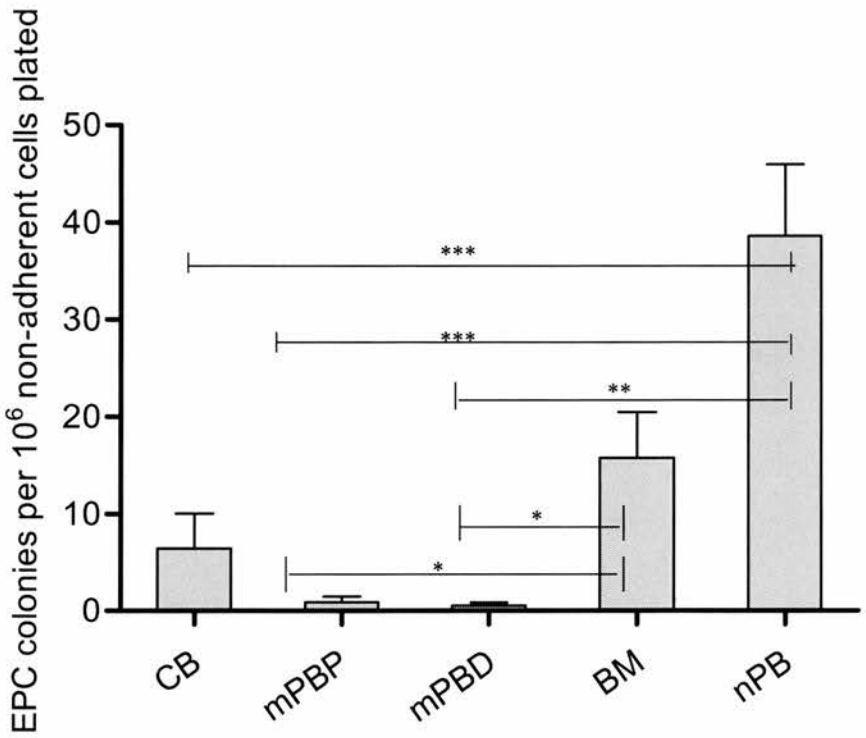


Figure 5.20 Endothelial progenitor cell colony assay (CFU-EPC) in haematopoietic stem cell clinical transplant sources and normal blood

Endothelial progenitor cell colonies (CFU-EPC) per 10^6 cells plated in the different sources tested. Normal peripheral blood (nPB)(n=15), bone marrow (BM)(n=7), cord blood (CB)(n=11) and G-CSF-mobilised peripheral blood samples (mPBP, patient (n=11); or mPBD, donor (n=5)). (* = $p<0.05$; ** = $p< 0.01$; *** = $p<0.001$, Mann-Whitney test)

5.2.3 (b) Endothelial progenitor cell colony forming unit (CFU-EPC) appearance in sources of haematopoietic stem cells and in normal peripheral blood

Where CFU-EPC from bone marrow, CB or mobilised peripheral blood sources were seen they were morphologically different and much smaller than those generated from peripheral blood mononuclear cells. All cord blood cells acquired spindle shaped morphology while most of those from G-CSF mobilised samples remained rounded, Figure 5.21

The CFU-EPC potential of the adherent cells normally discarded after the primary adherence step was also tested. None of the sources tested showed colony formation at the end of the 3 days nor were CFU-EPC seen even after 2 weeks in culture.

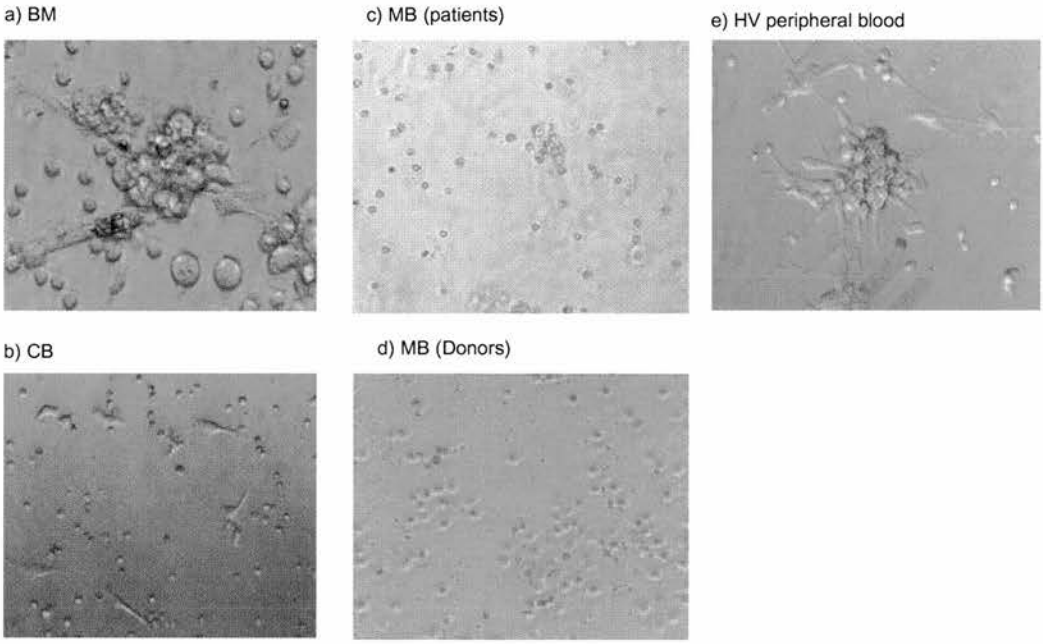


Figure 5.21 Images from endothelial progenitor cell colony assay cultures in different haematopoietic stem cell clinical transplant sources and normal blood

Representative phase contrast images from CFU-EPC assay cultures from (a) bone marrow, (b) cord blood, (c) mobilised peripheral blood (patients), (d) mobilised peripheral blood (donors) and (e) normal peripheral blood (healthy volunteer).

mean (SEM)						
Sources	BM	CB	mPBP	mPBD	nPB (HV)	Statitistically significant
total cells/ml (10^3)	1.63 (0.3)n=10	1.54 (0.4)n=21	1.41 (0.26)n=27	1.73 (0.6)n=5	1.17 (0.41)n=16	No significant difference
CD34+ (% of wbc)	0.71(0.1) n=10	0.42 (0.04)n=21	1.46 (0.36)n=27	0.28 (0.09)n=5	0.09 (0.02)n=16	BMv CB p**; CBv mPBP p*; BM v mPBP p**; BMv mPBD p**; CBv nPB p***; BMv nPB p***;mPBDv nPB p* ;mPBPv nPB p***
CD133+ (% of wbc)	0.46 (0.07) n=8	0.4 (0.05)n=7	1.04 (0.2)n=7	0.21 (0.1)n=3	0.07 (0.01)n=16	CBv mPBP p*; CBv nPB p***; BMv nPB p***; mPBPv nPB p***
VEGFR2 (% of wbc)	4.94 (1.69)n=10	9.94 (3.17)n=21	21.34 (5.23)n=27	4.5 (1.7)n=5	3.65 (1.97) n=16	mPBPv nPB p**
VEGFR2+ (% of CD34+)	5.43 (0.9)n=10	8.93 (1.2)n=21	3.92 (0.92)n=27	4.19 (2.9)n=5	24.49 (4.78)n=16	CBv mPBP p***; CBv mPBD p*; CBv nPB p*; BMv nPB p**; mPBPv nPB p***; mPBDv nPB p**
VEGFR2+ (% of CD133+)	0.43 (0.22)n=8	2.05 (1.35)n=7	2.4 (0.89)n=7	2.6 (2.5)n=3	23.79 (4.85)n=16	BMv mPBP p**; BM v nPB p**; CB v nPB p*; mPBPv nPB p*
CD45^{dim} (% of CD34+)	11.08 (1.86)n=10	15.08 (1.86)n=21	1.93 (0.39)n=27	1.81 (0.9) n=5	37.34 (5.61) n=16	BMv mPBP p***; CBv mPBP p***; BMv mPBD p**; CBv mPBD p**; CBv nPB p***; BMv nPB p***; mPBPv nPB p***; mPBDv nPB p**
CD45^{dim} (% of CD133+)	29.7 (9.4)n=8	2.14 (0.86)n=7	19.28 (13.2)n=7	NT	31.25 (10.72)n=16	BMv CB p*
CD133- (% of CD34+)	63 (5.65)n=8	35 (6.14)n=7	15.85 (5.5)n=7	21.22 (6.09)n=2	62.1 (3.96)n=16	CBv mPBP p*; BMv CB p**; BMv mPBP p***; CBv nPB p**; mPBPv nPB p***
CD133+ (% of CD34+)	13 (5.65)n=8	53 (6.14)n=7	79.76 (5.5)n=7	71.78 (6.09)n=2	11 (3.96)n=16	BMv mPBP p***; CBvmPBP p*; BMvCB p**; CBv nPB p**; mPBPv nPB p***
CD34- (% of CD133+)	31.2 (15.4)n=8	21.7 (9.6)n=7	9.13 (5.7)n=7	11.9 (11.4)n=2	33.1 (10.41)n=16	BMv mPBP p*; CBv nPB p*; mPBPv nPB p**
CD34+VEGFR2+(% of CD133+)	0.43 (0.22)n=8	2.05 (1.35)n=7	3.27 (0.89)n=7	2.59 (2.6)n=2	3.29(1.69)n=16	BMv mPBP p**
CD34-VEGFR2+(% of CD133+)	5.73 (2.85)n=8	2.33 (0.65)n=7	2.39 (1.81)n=7	5.17 (5.1)n=2	20.5 (4.07)n=16	BMv nPB p*; CBv nPB p*; mPBPv nPB p*
CD133-VEGFR2+ (% of CD34+)	4.47 (1.17)n=8	10.59 (1.58)n=7	0.89 (0.6)n=7	2.64 (2.2)n=2	20.42 (3.9)n=16	BMv CB p**; CBv mPBP p**; mPBPv BM p*; BMv nPB p*; mPBPv nPB p**
CD146+ CD3- (% of wbc) (CEC)	3.4 (0.71)=6	2.63 (0.46)n=4	0.55 (0.16)n=8	NT	0.62 (0.27) n=4	BMv mPBP p**; CBv mPBP p*; BMv nPB p*
EPC/cells plated (10^6)	15.77 (4.7)n=7	6.46 (3.6)n=11	0.91 (0.58)n=11	0.6 (0.4) n=5	39.37 (8.72)n=15	BMv mPBP p*; BMv mPBD p*; CBv nPB p***; mPBPv nPB p***; mPBDv nPB p**

Table 5.4 Phenotype analysis by proportions in the different sources tested.

Surface expression of a panel of markers was analysed in the different sources which include bone marrow (BM), cord blood (CB), G-CSF-mobilised blood for autologous transplant (mPBP), G-CSF mobilised blood for allogeneic transplant (mPBD) and healthy normal donors (nPB). Statistically significant relationship was considered if the p value was <0.05 ($p<0.05$=*, $p<0.01$=** and $p<0.001$=***). The different sources were compared using non-parametric tests (Mann-U Whitney, U). Results are expressed as the mean (Standard error); n=number of samples tested, NT= not tested.

Sources	BM	CB	mPBP	mPBD	nPB (HV)	Statitistically significant
No CD34$^+$ cells/ml (103)	115	64.68	205.8	48.44	10.53	BMv CB p*; BMv nPB p***; CBv nPB p***;mPBPv nPB p***; mPBDv nPB p*
No CD133$^+$ cells/ml (10^6)	74.9	61.6	146.6	36.33	8.19	BMv CB p*; BMv nPB p***; CBv mPBP p*; mPBPv nPB p***
No CD34$^+$ VEGFR2+ cells/ml (103)	6.24	5.77	8.06	2.02	2.57	BMv mPBD p**; BMv nPB p**; CBv mPBD p**; CBv nPB p**
No CD133$^+$ VEGFR2+ cells/ml (10^6)	0.32	1.26	3.51	0.94	1.94	CBv mPBP p**;CBvnPB p*, BMv mPBP p***

Table 5.5 Phenotype analysis by numbers in the different sources tested.

Total number of CD34$^+$ cells, CD133$^+$ cells, CD34$^+$ cells co-expressing VEGFR2 and CD133$^+$ cells co-expressing VEGFR2 analysed in the different sources tested. Statistically significant relationship was considered if the p value was <0.05 ($p<0.05$=*, $p<0.01$=** and $p<0.001$=***). The medians of the different sources were compared using non-parametric tests (Mann-U Whitney, U). Results are expressed by the mean (Standard error).

Characterisation of endothelial progenitor cell colony forming (CFU-EPC) potential in subpopulations of mononuclear cells from haematopoietic stem cell sources and normal peripheral blood

5.2.3.1 Flow cytometry characterisation of the cells used for the CFU-EPC assay

A flow cytometry phenotype characterisation was performed on the cells which are used in the final phase of the colony assay for endothelial progenitors (CFU-EPC). These are the mononuclear cells (MNC) which remain non-adherent after the 2-day of primary culture step on fibronectin-coated plates. The MNC sources (see Figure 5.20 and Table 5.4) which were demonstrated to generate CFU-EPC (normal peripheral blood and bone marrow) were compared with those sources which generate few or no CFU-EPC (cord blood and G-CSF-mobilised peripheral blood), for expression of a range of surface markers CD45, CD34, CD14, CD3, CD16, CD133, VEGFR2, VE-cadherin, CD29, CXCR4, and GP1a.

The number of non-adherent leucocytes recovered per ml of MNC sample was similar in all the different sources analysed (~$2x10^6$ wbc/ml). Equally, similar CD45 expression was detected in all sources although the percentage of $CD45^+$ cells was lower in cord blood and mobilised peripheral blood sources, probably due to the presence of immature CD45-negative red cells, resistant to lysis (in flow cytometry protocols), which frequently contaminate buoyant-density MNC isolates from cord and mobilised blood. Percentages of $CD45^+$ cells (gated in FSC/SSC) and percentages of $CD34^+$, $CD133^+$, $VEGFR2^+$, VE-$cadherin^+$, $CD29^+$, $CXCR4^+$, $CD3^+$, $CD14^+$ and $CD16^+$ (gated on $CD45^+$ (PercP bright) population) are shown below (Table 5.6). $VEGFR2^+$ cells as a fraction of the $CD34^+$ cells and $CD34^+$ cells co-expressing VEGFR2 as a fraction of the $CD133^+$ cells are also illustrated in table 5.6. As expected bone marrow, cord blood and mobilised peripheral blood sources had significantly higher numbers of $CD34^+$ and $CD133^+$ HSCs by comparison with normal peripheral blood. It was also noted that mobilised peripheral blood samples had a notably lower number of $CD3^+$ cells (T-lymphocytes) and higher numbers of total $VEGFR2^+$ cells by comparison to the other sources. These were evidently not VEGFR2-expressing HSC, since $CD34^+VEGFR2^+$ or $CD34^+CD133^+VEGFR2^+$ EPC phenotypes were significantly higher in normal peripheral blood and bone marrow by comparison with cord blood and mobilised peripheral blood samples. No substantial differences in the number of $CD14^+$, $CD16^+$, or VE-$cadherin^+$ cells were seen between the different sources (Table 5.5).

	wbc (10^6/ml)	% CD45+ (gated on FCS/SSC)	%CD34+	%CD133+	%VEGFR2+	%VE-cadherin+	%CD29+	%CXCR4+	%VEGFR2+ (gated on CD34+ cells)	%CD34+VEGFR2+ (gated on CD133+ cells)	%CD3+	%CD14+	%CD16+
CB	2.27	44.34	1.15	0.7	4.11	4.66	5.09	28.17	19.89	10.72	23.29	5.05	8.66
MB	2.21	49.62	1.02	1.01	20.67	2.63	9.55	30.51	16.73	8.9	4.28	19.67	1.29
BM	2.3	72.22	0.84	0.49	8.15	6.74	53.96	47.66	46.83	12.13	22.06	13.63	4.52
PB	2.29	64.47	0.18	0.06	7.32	4.63	51.31	49.6	33.73	30	38.95	8.41	6.97

Table 5.6 Phenotype characterisation of cells which are non-adherent on fibronectin-coated plates after the 2-day primary culture of the CFU-EPC assay

Flow cytometry characterisation of the non-adherent cells after 2-day primary CFU-EPC culture of MNC isolated from different putative EPC sources. Sources were compared for expression of a range of different surface markers. White blood cells were expressed at 10^6/ml

5.2.3.2 Expression of CD29 (ß-1 integrin) in cells used for the CFU-EPC assay

Figure 5.22a shows the percentage these CFU-EPC primary culture (fibronectin non-adherent) cells expressing CD29 (ß-1 integrin). The percentage of $CD29^+$ cells was much lower in sources which did not generate EPC colonies (cord blood and mobilised peripheral blood sources) by comparison with those which did produce CFU-EPC (normal peripheral blood and bone marrow) (cord blood v mobilised peripheral blood p=0.03; cord blood v bone marrow p=0.001 and mobilised peripheral blood v bone marrow p=0.015, Mann-Whitney test) (BM n=5, CB n=9, mPBP n=6, mPBD n=2 and nPB n=6). Within the $CD29^+$ population (gated in flow cytometry), in all sources, no difference was seen in frequency of co-expression of VEGFR2 or VE-Cadherin.

5.2.3.3 Expression of CXCR4 (SDF-1) in cells used for the CFU-EPC assay

The percentage of cells (fibronectin non-adherent) staining positive for CXCR4 was significantly lower in the sources which did not generate EPC colonies (cord blood and mobilised peripheral blood) compared to those which did produce CFU-EPC (normal peripheral blood and bone marrow) (Figure 5.22b) (cord blood v normal peripheral blood p=0.0016, mobilised peripheral blood v normal peripheral blood p=0.031, Mann-Whitney test) (BM n=5, CB n=9, mPBP n=6, mPBD n=2 and nPB n=6). No differences between sources were seen of the co-expression of $CXCR4^+CD34^+$ or $CXCR4^+CD133^+$. Percentage of $CD34^+$ cells co-expressing CXCR4 ((47.4 CB)(47.8 BM)(50.1 mPBP)(66.4 mPBD) and (65.6 nPB)). Similarly percentage of $CD133^+$ cells co-expressing CXCR4 ((51 CB)(81.62 BM)(76.9 mPBP)(38.9 mPBD) and (75.22 nPB)).

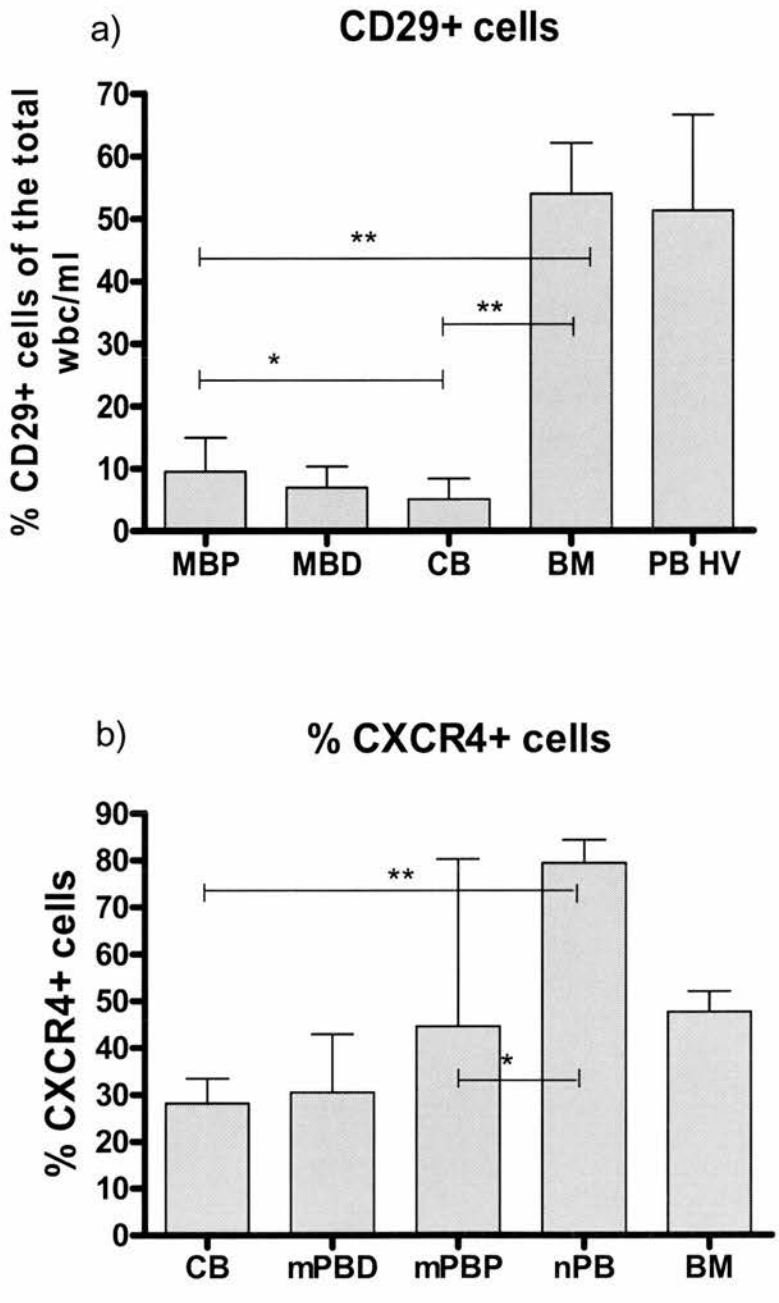


Figure 5.22 CD29 and CXCR4 expression on cells which are non-adherent on fibronectin-coated plates after the 2-day primary culture of the CFU-EPC assay

Non-adherent cells after 2-day primary CFU-EPC culture of MNC from different sources were analysed and compared for the expression of CD29 (a) and for the expression of CXCR4 markers (b).

5.2.3.4 Effect of normal peripheral blood selective mononuclear cell subpopulation enrichments on CFU-EPC frequency.

5.2.3.4.1 Depletion/enrichment of CD34$^+$ cells

Peripheral blood CD34$^+$ cells were enriched from MNC by magnetic bead separation, and the CD34-negative population was also recovered, and the two populations were plated separately in a 24-well fibronectin-coated plate in complete endothelial culture medium (CECM) (Stem cell Technologies, UK). After 5 days of culture the number of spindle-shaped cells and the number of CFU-EPC colonies were counted by microscopy. The CD34$^+$ enriched cell fraction, with an average of 65% CD34$^+$ purity, did not produce CFU-EPC, suggesting that these cells are probably not directly the source of CFU-EPC. After 5 days the cells remained very small and rounded (Figures 5.23a and 5.24) and after extended cultures started to die. By contrast the CD34 depleted fraction contained healthy spindle-shaped cells and did generate some CFU-EPC, though at a much lower frequency than did unseparated MNC (Figure 5.23b and 5.24).

5.2.3.4.2 Depletion/enrichment or depletion of CD133$^+$cells

Peripheral blood CD133$^+$ cells were magnetically separated from the CD133 negative population and the two populations were plated separately in a 24-well fibronectin plate with complete endothelial culture medium (CECM) (Stem cells Technologies, UK). The CD133$^+$ fraction (average of 70% purity), unlike CD34$^+$ fraction (above), did acquire 100% spindle-shaped cell morphology. These cells appeared to expand but did not generate localised CFU-EPC colonies (see methods, described in chapter 2 section 2.4.1.2). After 12 days in culture the cells started to die, probably due to the exhaustion of cytokines in the media. The CD133-depleted fraction behaved very similar to the CD34-depleted fraction (above) and gave spindle-shaped cells, which generated some CFU-EPC colonies though at a much lower frequency than did unseparated MNC (Figure 5.23c and d and 5.24).

5.2.3.4.3 Cell-enrichment by adherence to tissue-culture plastic.

Peripheral blood MNCs were allowed to adhere to plastic for 2 hours in a 25cm^2 tissue culture flask in IMDM medium. Cells remaining non-adherent after 2h were recovered, then the plastic adherent cells were recovered by desorption. 81% of the 2h plastic adherent cell population were CD14 positive. The non-adherent cells did not generate CFU-EPC colonies, whereas adherent cells had the capacity to generate very large and characteristic CFU-EPC colonies at a much higher frequency than

did unseparated MNC. (Figure 5.23e and f and 5.24). Thus, it appears that all CFU-EPC are located in the plastic-adherent population from MNC.

5.2.3.4.4 CD14-positive cells are responsible for CFU-EPC, not CD14-negative cells.

Peripheral blood MNCs following 2h plastic adherence were magnetically separated or sorted by flow cytometry for expression of CD14, giving an average purity of 84% and 98%, respectively. Both $CD14^+$ and CD14 negative cells were recovered and plated in 24-well fibronectin-coated plates in CECM. After 5 days culture, only the $CD14^+$ cells from the plastic adherent MNC had generated CFU-EPC colonies (Figure 5.23g and 5.24). No colonies were seen in wells containing CD14-negative cells from the plastic adherent MNC (Figure 5.23h and 5.24). Thus, it appears that all CFU-EPC are located in the CD14-positive population of the plastic-adherent population from MNC

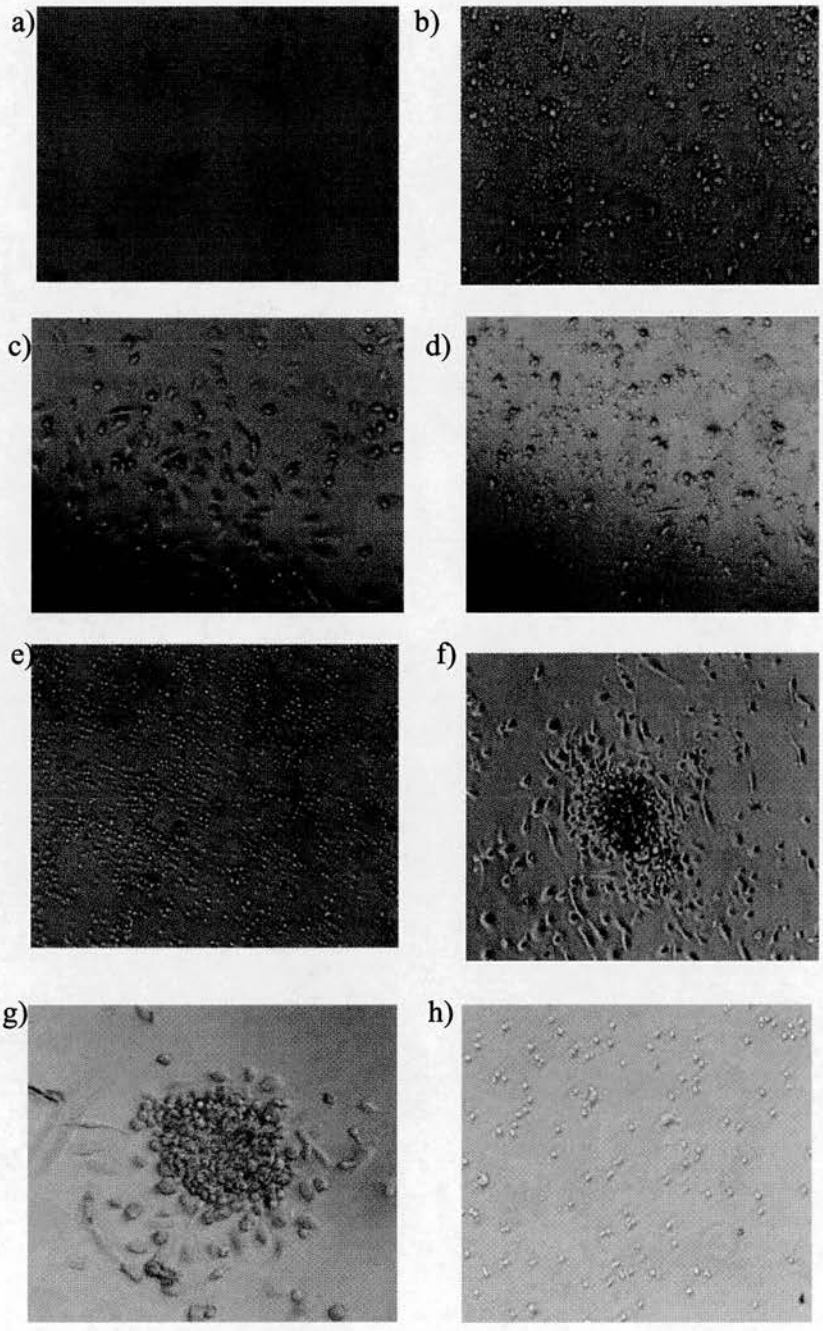


Figure 5.23 CFU-EPC detection following selective cell-enrichments from normal peripheral blood mononuclear cells. Phase contrast images

Phase-contrast images showing CFU-EPC and/or cell morphology of sub-populations of cells isolated from peripheral blood MNC. a) CD34-enriched cells, b) CD34-depleted cells, c) CD133-enriched cells, d) CD133-depleted cells, e) cells not adherent to plastic after 2h, f) 2h plastic adherent cells, g) CD14+ FACS-sorted cells, h) CD14- FACS-depleted cells.

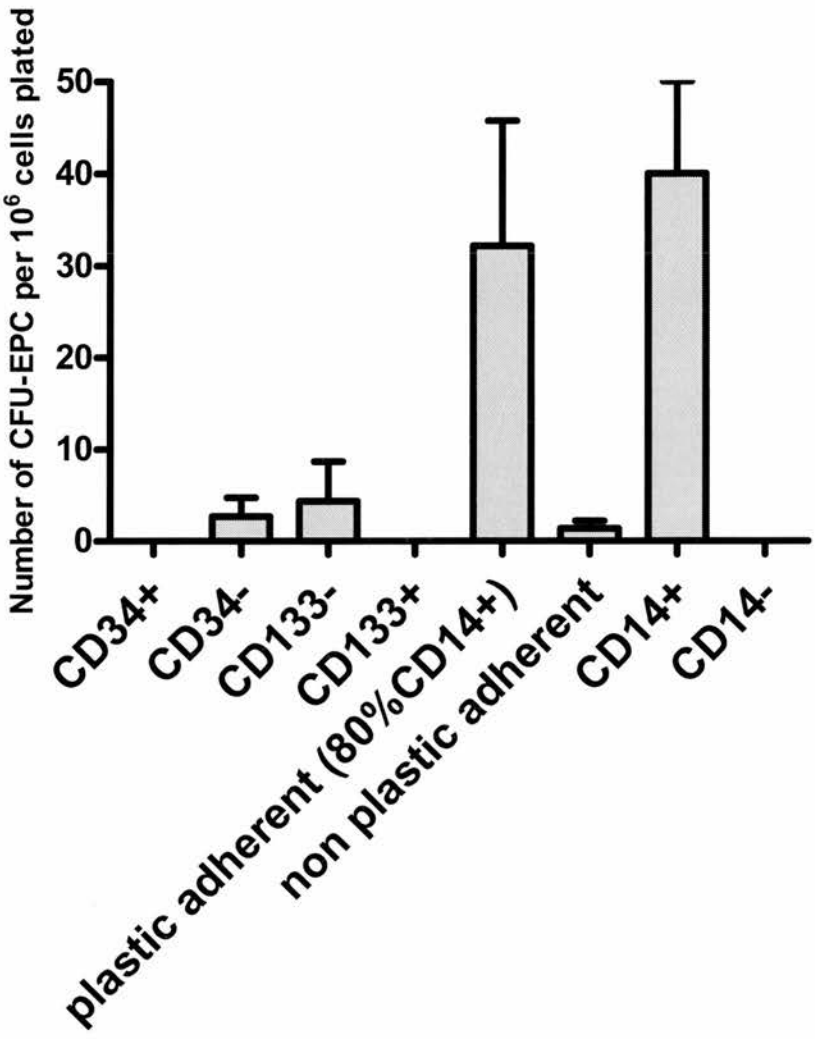


Figure 5.24 Frequency of CFU-EPC detected following selective cell-enrichments from normal peripheral blood mononuclear cells.

Number of CFU-EPC derived from $CD34^+$ cells, $CD34^-$ cells, $CD133^+$ cells, $CD133^-$ cells, 2h plastic adherent cells, 2h plastic-non-adherent cells, $CD14^+$ FACS-sorted cells and $CD14^-$ FACS-depleted cells were represented in this graph.

5.2.3.5 Immunostaining and flow cytometry of the CFU-EPC and accompanying cells.

Spindle-shaped cells and CFU-EPC generated from peripheral blood $CD14^+$ sorted cells were brightly positive for expression of DiI-Ac-LDL and retained CD14. These cells also expressed CD45, VE-cadherin and the receptor for u*lex europaeus* agglutinin-1 (UEA-1), though they did not express CD34 (Figure 5.25). Flow cytometry results showed (in Figure 5.26) that increasing time of culture (up to 10 days) results in a concomitant down-regulation of expression of CD45, SDF-1 and CD14, accompanied by up-regulation of VEGFR2 and CD146, presumably as cells differentiate along an endothelial pathway. Expression of VE-cadherin, CD34 and CD29 did not change significally.

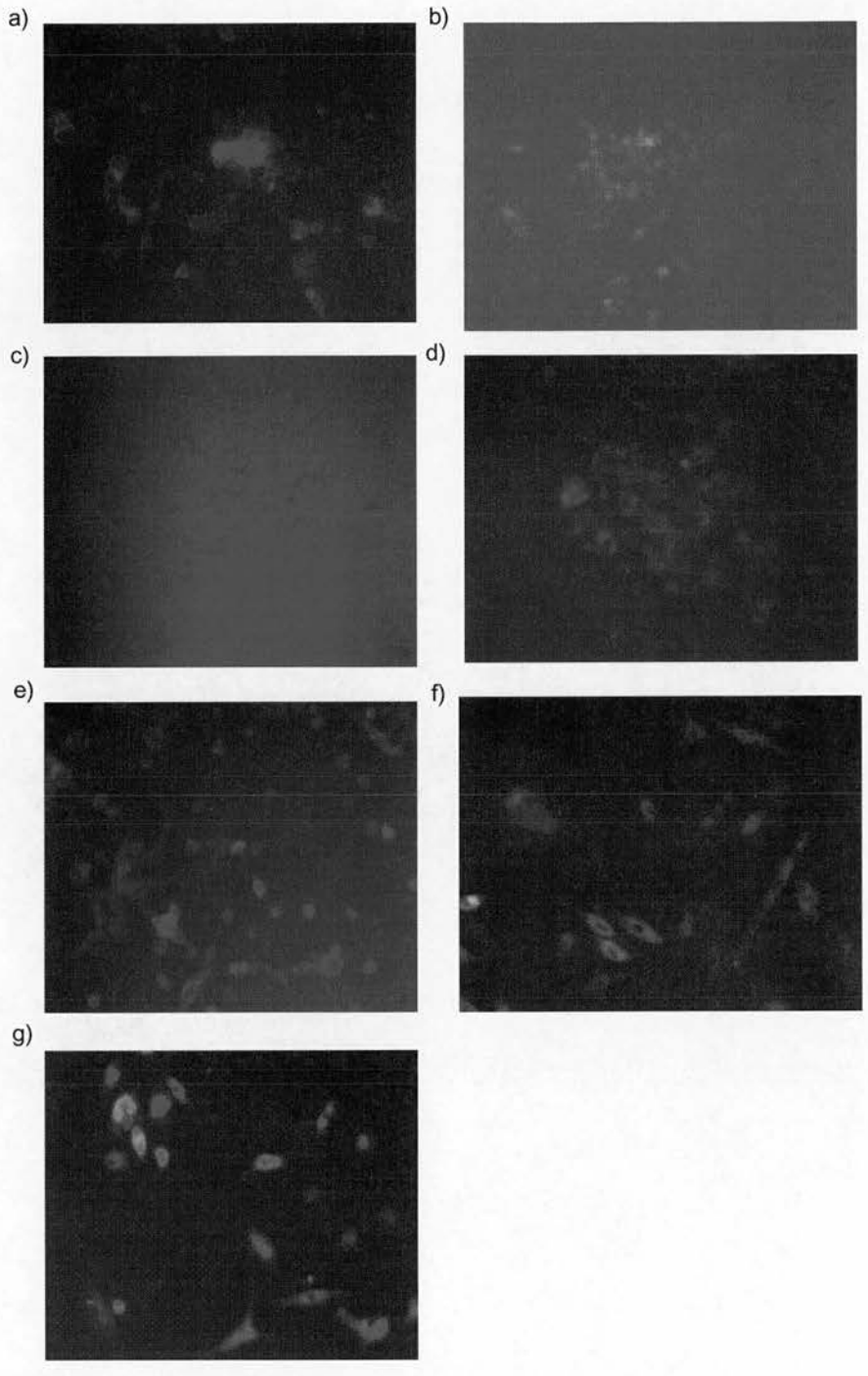


Figure 5.25 Immunostaining of cells in CFU-EPC assay cultures

Fluorescence microscopy images of immunohistology staining of CFU-EPC (a-d) and spindle-shaped cells (e-g) derived from peripheral blood: (a and e) CD45 staining, (b and f) double positive of DiI-Ac-LDL (green) and CD14 PE (red), c) CD34-FITC staining (most of the cells were CD34 negative), g) DiI-Ac-LDL uptake, d) double stain of UEA-1 FITC (green) and VE-cadherin PE (red).

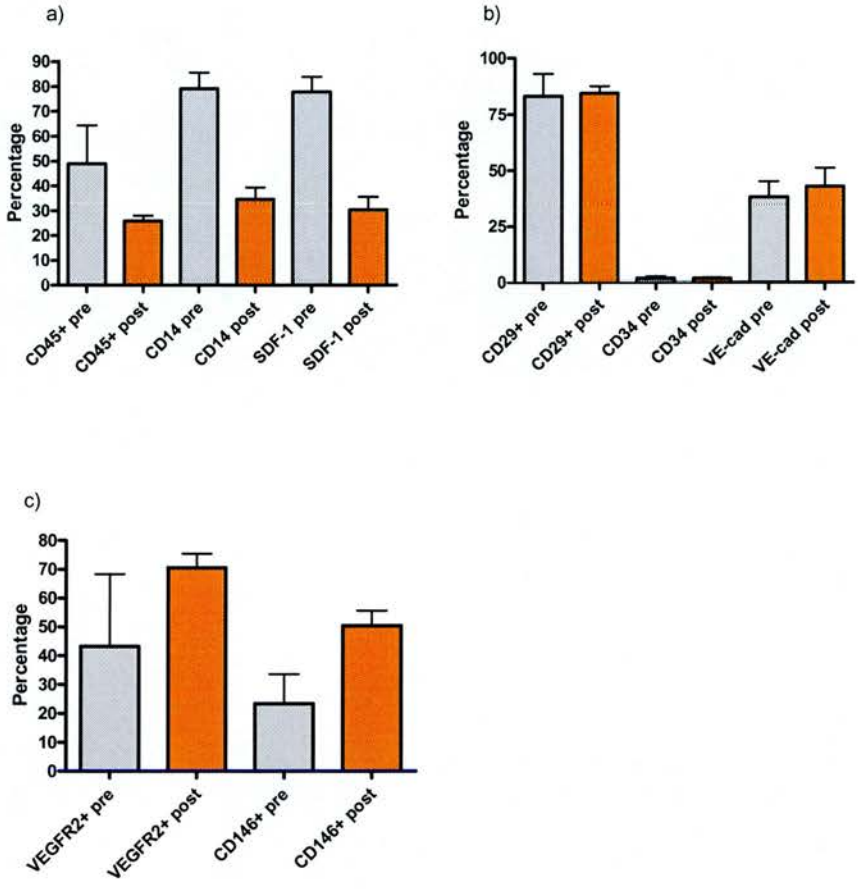


Figure 5.26 Comparison of the percentage of a panel of different surface markers before and after CFU-EPC culture

Changes in expression of surface markers by 2h plastic adherence $CD14^+$ peripheral blood derived cells before (grey) and recovered following 10 day culture on fibronectin in the CFU-EPC assay medium (orange). Results shown: panel a) down-regulation, b) no change and c) up-regulation of markers.

5.2.3.6 Which subset of the CD14⁺ cells?

CD14-positive cells are increasingly recognised as functionally heterogeneous, but it is not known whether they are polyfunctional or whether different functions derive from discrete subpopulations of CD14-positive cells. 2h plastic adherent peripheral blood $CD14^+$ cells gave an average of 40 CFU-EPC colonies/10^6 cells plated, giving a frequency of about 1 cell out of 25,000 $CD14^+$ cells plated being able to proliferate to generate CFU-EPC in culture. Phenotypically distinct subsets of $CD14^+$ cells were isolated by flow cytometry sorting and subjected to the CFU-EPC assay in order to further define the phenotype of the cell which generates CFU-EPC (Figure 5.27 a-d).

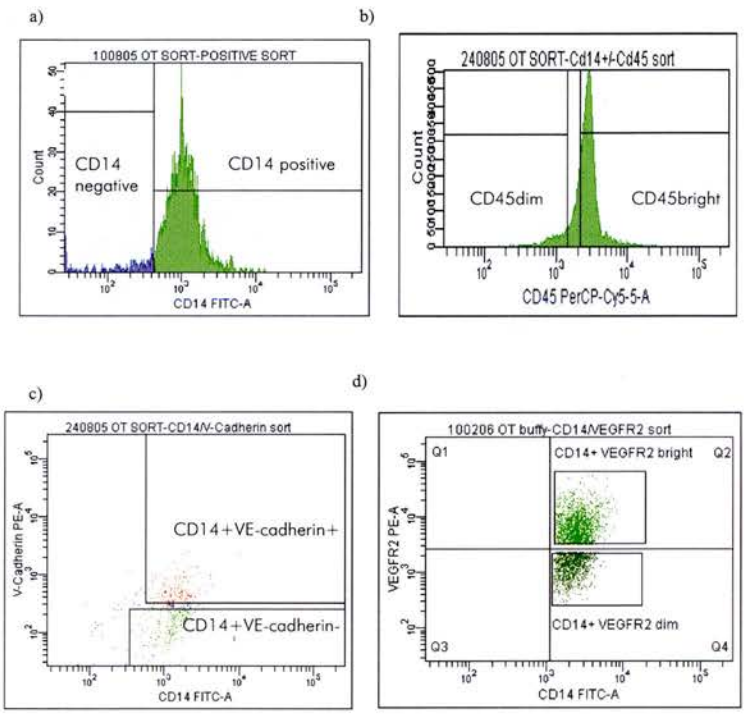


Figure 5.27 Regions set for sorting $CD14^+$ cell subsets by flow cytometry

Cell sorting populations, a) $CD14^+$ and $CD14^-$ cell sort. b) CD45-bright and CD45-dim staining, c) $CD14^+$ VE-cadherin-positive cells and $CD14^+$ VE-cadherin-negative cells, d) $CD14^+$ VEGFR2-bright cells and $CD14^+$ VEGFR2-dim cells. Gates were set by reference to appropriate unstained controls.

5.2.3.6.1 CFU-EPC frequency in CD45-bright and CD45-dim cell subpopulations of CD14-positive cells

2h plastic adherent peripheral blood $CD14^+$ cells were sorted into CD45-bright and CD45-dim subpopulations by flow cytometry and plated separately in fibronectin-coated 24-well plates in CECM for at least 5 days. Both subpopulations of the $CD14^+$ cells generated CFU-EPC colonies at a comparable frequency (Figure 5.28). However, after an extended 14 days of culture 100% of the surviving cells in the $CD14^+CD45^{dim}$ fraction acquired a spindle-shaped morphology, whereas only about 70% of the surviving cells in the $CD14^+CD45^{bright}$ fraction showed the same morphology. Some cells in the $CD14^+CD45^{bright}$ fraction became detached and died.

5.2.3.6.2 CFU-EPC frequency in VE-Cadherin+/- cell subpopulations of CD14-positive cells

2h plastic adherent peripheral blood $CD14^+$ cells were fractioned by flow cytometry sorting into VE-Cadherin-positive or VE-cadherin-negative cell populations. Following 5 days culture in CECM in fibronectin coated plates both fractions generated CFU-EPC colonies. No significant difference in frequency was seen between the two fractions (Figure 5.28).

5.2.3.6.3 CFU-EPC frequency in VEGFR2+/- cell subpopulations of CD14-positive cells

Similarly, following 5 day culture in CECM on fibronectin coated plates no significant difference in CFU-EPC numbers was seen between $VEGFR2^+$ or VEGFR2-negative fractions of 2h plastic adherent $CD14^+$ peripheral blood mononuclear cells (Figure 5.28).

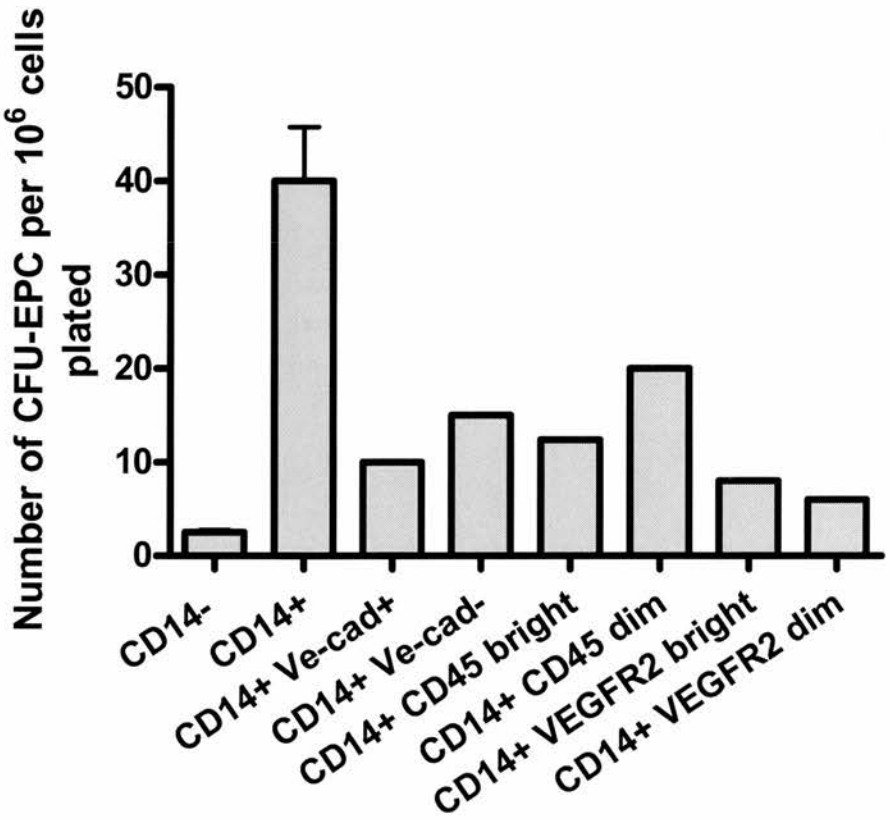


Figure 5.28 CFU-EPC frequency in CD14-negative, CD14-positive and in various subsets of CD14-positive plastic-adherent MNC sorted by flow cytometry

Number of CFU-EPC per 10^6 cells plated obtained from the different cell sorted populations; $CD14^+$ and $CD14^-$, $CD14^+CD45^{bright}$ and $CD14^+CD45^{dim}$, $CD14^+$VE-cadherin$^+$ cells and $CD14^+$VE-cadherin$^-$ cells, d) $CD14^+VEGFR2^{bright}$ cells and $CD14^+VEGFR2^{dim}$ cells.

5.2.3.7 Flow cytometry analysis of the different CD14-positive subfractions in whole blood between all putative EPC sources.

Although CFU-EPC potential differed dramatically between sources, proportions of $CD14^+$ cells remained unchanged in the different putative EPC sources. It is probable that not all $CD14^+$ cells have this proliferative colony forming capacity. Therefore different analysis of $CD14^+$ subsets were repeated for all the putative EPC sources studied (Table 5.7). No significant difference was seen between peripheral blood and all the other sources studied for co-expression of $CD14^+$ and the panel of antigens tested. The only subsets where normal peripheral blood was significally different to other sources were $CD14^+$VE-cadherin$^+$ cells (nPB v CB p=0.009) where normal peripheral blood had a lower proportion compared to cord blood, and $CD14^+CD29^+$ cells (nPB v BM p=0.03, nPB v CB p=0.01 and nPB v mPB p=0.01) where normal peripheral blood had also a lower proportion of $CD14^+CD29^+$ cells compared to the others. However no clear conclusion could be drawn to associate these phenotypes with CFU-EPC potential.

Sample ADHERENT	CD14+CD45+	CD14+VEGFR2+	CD14+VE-CAD+	CD14+CD29+	CD14+CXCR4+	CD14+CD34+
average CB	95.71	53.08	24.42	99.47	49.26	1.74
average mPB	86.75	28.28	19.81	98.67	87.60	1.45
average BM	84.75	63.93	24.18	95.32	58.52	2.90
average nPB	99.00	52.84	4.55	67.99	82.84	0.37

Table 5.7 Phenotype characterisation of $CD14^+$ cells

2h plastic adherent cells $CD14^+$ cells from all-putative EPC sources studied were analysed for co-expression of a panel of endothelial and haematopoietic markers; CD45, VEGFR2, VE-Cadherin, CD29, CXCR4 and CD34. (BM n=5, CB n=9,mPBP n=5, mPBD n=2 and nPB n=6).

5.2.3.8 Flow cytometry characterisation of plastic adherent cells

Flow cytometry phenotype characterisation of the 2h plastic adherent population was carried out, and a comparison between peripheral blood, bone marrow, cord blood and G-CSF mobilised sources was made (Table 5.8). The percentage of $CD45^+$ cells in 2h plastic adherent cells from peripheral blood and bone marrow was lower than that seen in cord blood and mobilised blood. Peripheral blood contained lower percentage of $CD16^+$ cells compared to the other sources. It maybe indicates that peripheral blood contains less $CD16^+$ immature neutrophils, which could be retained after Ficoll MNC separation in the other sources. Moreover peripheral blood contained slightly more VE-cadherin$^+$ and $CD14^+$ cells than all other sources tested but no other significant differences were seen.

Sample ADHERENT	CD45+	CD34+	% CD133+	% VEGFR2+	% Ve-cad+	CD29+	SDF-1+	CD34+VEGFR2+	CD34+VEGFR2+CD133+	CD3	CD14	CD16	CEC
average CB	75.42	1.27	0.24	37.27	20.12	92.64	59.39	100.00	37.50	11.11	55.93	17.85	5.14
average mPB	94.83	1.83	0.25	21.12	9.74	78.17	82.41	73.97	31.94	3.13	51.09	18.18	20.56
Average BM	62.47	4.39	1.44	39.94	17.91	46.20	48.00	68.28	74.04	6.69	41.14	29.26	26.29
average nPB	65.26	1.98	1.18	43.20	42.88	83.19	77.79	87.75	36.00	4.98	79.08	0.03	13.19

Table 5.8 Phenotype characterisation of 2h plastic adherent cells

Characterisation of 2h plastic adherent cells by flow cytometry using a panel of antibodies specific for endothelial and haematopoietic cells.

5.2.3.9 Studies of reduced frequency CFU-EPC in cord blood and G-CSF-mobilised peripheral blood MNC.

For comparison with results obtained using peripheral blood, selective enrichments were also performed using cord blood and G-CSF mobilised blood. In both cases, none of the $CD34^+$ or $CD133^+$ enriched populations were able to generate CFU-EPC colonies (Figure 5.29). However, 2h plastic adherent cells from cord blood and mobilised peripheral blood (75% and 74% $CD14^+$ respectively) did generate some CFU-EPC, but they were less frequent and not of the same size as those from peripheral blood 2h plastic adherent cells (Figure 5.29).

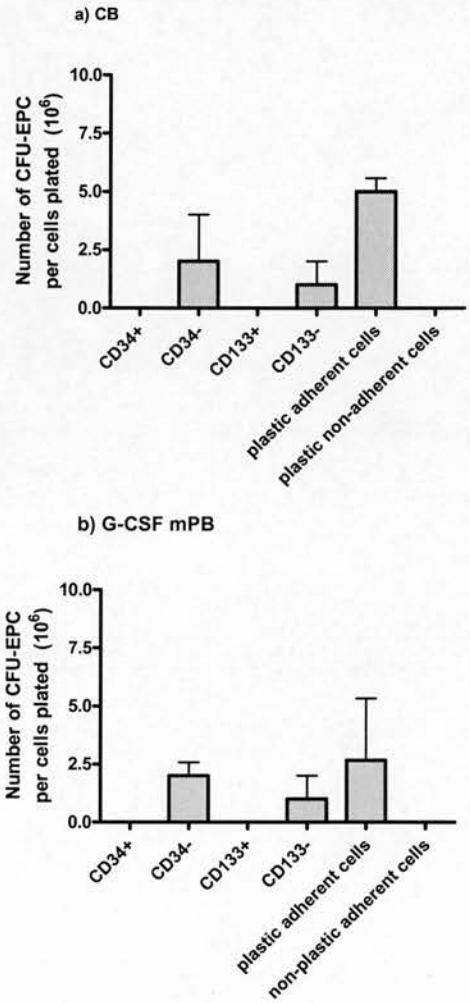


Figure 5.29 CFU-EPC frequency in mononuclear cell subpopulations enriched from cord blood and mobilised peripheral blood.

Number of CFU-EPC derived from CD34-enriched cells, CD34-depleted cells, CD133-enriched cells, CD133-depleted cells, 2h plastic adherent cells, 2h plastic-non-adherent cells. From a) umbilical cord blood and b) G-CSF mobilised peripheral blood.

5.2.3.9.1 Has cord blood inhibitory factors in its plasma to block the endothelial colony capacity?

To test the hypothesis that the low frequency of CFU-EPC seen in cord blood samples was due to inhibitory factors, increasing volumes of cord blood-derived plasma was added to wells of peripheral blood non-adherent cells. Increasing plasma concentration did correlate with a reduction in CFU-EPC (Figure 5.30). To rule out the possibility that complement components in the plasma were responsible for the effect the titration was repeated using plasma heated to 56C for 30 minutes to inactivate complement activity. No difference was seen in CFU-EPC generation using native or heat-inactivated cord blood plasma (data not shown). Further investigation showed that inhibition of CFU-EPC by increasing concentration of plasma was not specific to cord blood-derived plasma. Increasing concentrations of peripheral blood-derived plasma from healthy donors also reduced CFU-EPC formation (Figure 5.30).

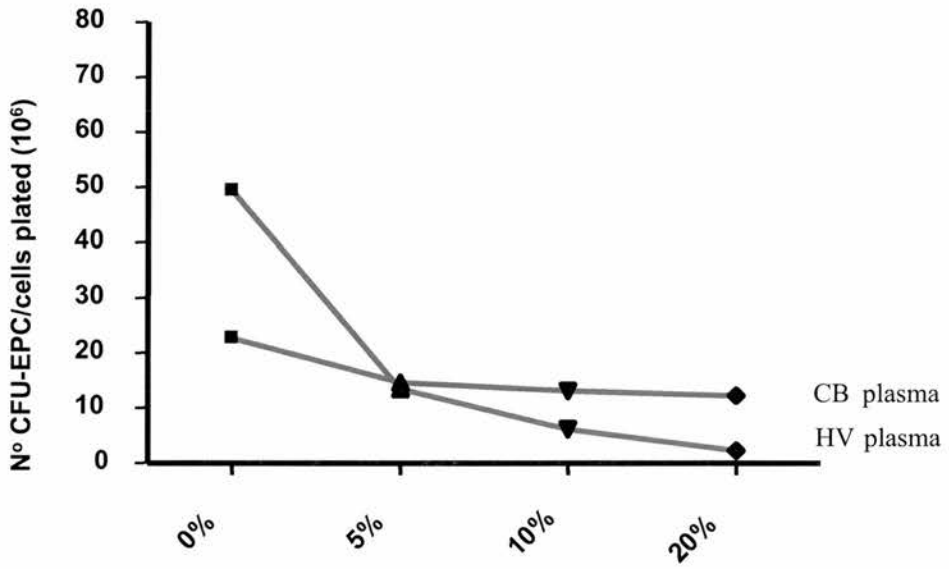


Figure 5.30 Effect of addition of cord blood plasma on assay of CFU-EPC frequency in peripheral blood MNC

Non-adherent peripheral blood cells after 2-day primary culture were plated. The number of CFU-EPC per cells plated were counted in situations of increasing concentrations of plasma (0%, 5%, 10% and 20%) added to the CFU-EPC assay cultures. Cord-blood-derived plasma (orange) and peripheral blood plasma from a healthy volunteer- derived plasma (magenta).

5.2.3.9.2 Primitive erythrocytes present in cord blood and mobilised blood MNC cultures

As both cord blood and mobilised peripheral blood have higher numbers of immature erythrocytes by comparison to normal peripheral blood or bone marrow, it was possible that these may have an effect on colony formation. Cord blood samples lysis-treated by a short isotonic shock, were compared to non-lysed cord blood samples. Results showed no difference in terms of number of CFU-EPC (data not shown). In most of these samples isotonic shock was not enough for the exclusion of all red cells, especially primitive red cells. An alternative method was tried, where cord blood MNC were layered over a diluted Ficoll-Histopaque (1.057 density), and centrifuged for 20 minutes at 500g. The cells floating above the Ficoll fraction were the less dense cells such as red cells and platelets; all the leucocytes should have sedimented in to the pellet. Such samples did not show any CFU-EPC frequency difference either (data not shown).

5.2.3.10 Effect of G-CSF addition *in vitro* on peripheral blood MNC CFU-EPC frequency

Low CFU-EPC generation was consistently observed in mobilised peripheral blood in both autologous patients (mPBP) and allogeneic donors (mPBD), which were virtually incapable of CFU-EPC formation (mPBP: 0.9 CFU-EPC/10^6 cells plated; mPBD: 0.6 CFU-EPC/10^6 cells plated). To investigate whether this was a consequence of a direct effect of the administered G-CSF, exogenous G-CSF was added to CFU-EPC assay cultures at a final concentration of 100ng/ml (the concentration optimal for *ex vivo* neutrophil differentiation from $CD34^+$ cells, see Chapter 3). Control wells received an equal volume of CECM without supplement. The results from 3 experiments showed a dramatic decrease in CFU-EPC number in the presence of exogenous G-CSF (Figure 5.31).

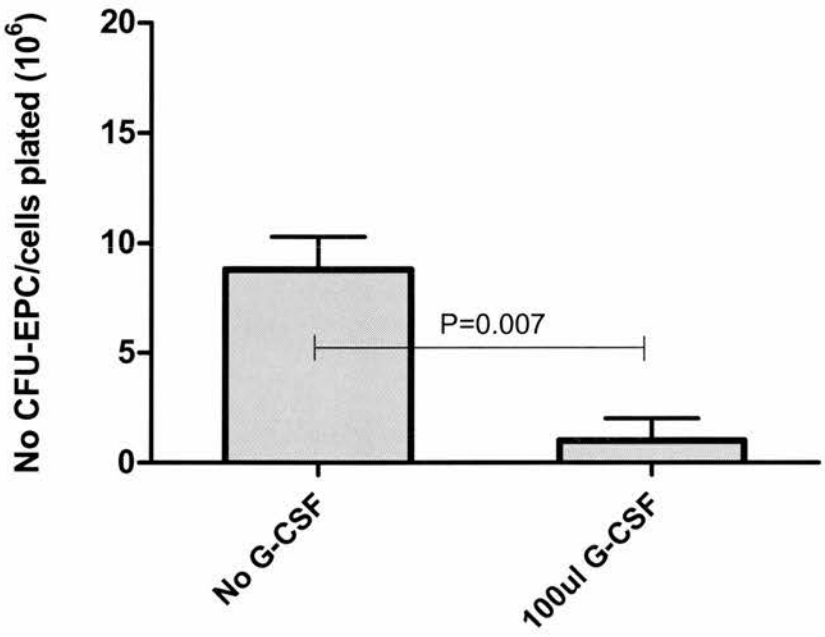


Figure 5.31 Effect of addition of G-CSF to the CFU-EPC assay

100ng/ml G-CSF in complete endothelial culture medium (CECM) was added to the non-adherent peripheral blood cells after 2-day primary culture to assess CFU-EPC potential. Control wells received an equal volume of CECM without supplement.

5.2.3.10.1 Effect of G-CSF administration on healthy individuals' peripheral blood CFU-EPC frequency

To confirm that G-CSF is directly modulating CFU-EPC generation, longitudinal peripheral blood samples were obtained before (pre) G-CSF administration, at PBSC apheresis harvest (peri) and at 2 months follow-up (post). Previously studies samples (above) were obtained at the time of PBSC apheresis harvest, which corresponds to the "peri" samples in this study. Colony formation was drastically reduced ($p=0.02$) (Mann-Whitney test, $n=4$) after G-CSF administration (26.72 CFU-EPC/10^6 cells plated & 0.67/10^6 cells plated CFU-EPC average respectively) (Figure 5.32). In samples taken 2 months after completion of G-CSF treatment (follow-up) CFU-EPC generation potential was comparable to that seen before commencement of G-CSF administration.

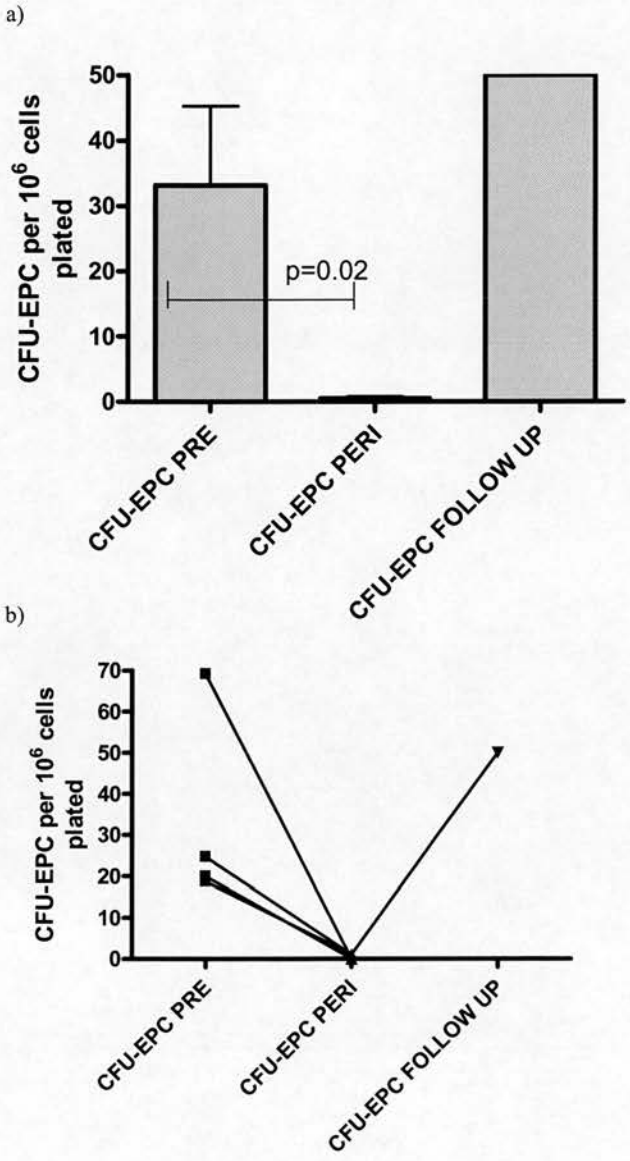


Figure 5.32 Effect of G-CSF administration on CFU-EPC generation in a longitudinal study

Colony formation was assessed in peripheral blood samples obtained from the same healthy donors (for allogeneic PBSC transplant) pre-, peri- and post G-CSF administration (follow up) (pre n=4, peri n=4 and post n=1). a) histogram and b) graph with connected dots for each individual. Data was statistically analysed using Wilcoxon paired test. The changes between pre and peri samples were not significant probably due to the low number of samples analysed. Stats were not used to post (follow-up) samples because of the only one sample analysed). Further recruitment of samples is under study.

5.2.3.10.2 Effect of G-CSF administration on healthy individuals' peripheral blood putative EPC phenotype frequencies

Phenotyping by flow cytometry did not show any difference between the 3 sampling points which could be associated with the alteration in CFU-EPC potential during G-CSF administration. Following G-CSF administration cell populations expressing $CD34^+$, $CD133^+$, and $VEGFR2^+$ alone or in combination; i.e. $CD34^+CD133^+$ or $CD34^+CD133^+VEGFR2^+$ increased by comparison with the same samples before G-CSF administration. $CD45^+$, VE-cadherin$^+$, $CD14^+$ and other marker combinations remained unchanged (Figure 5.33 and table 5.9). A reduction in $CD34^+CD45^{dim}$, $CD14^+$VE-cadherin$^+$, and $CD29^+$ cell populations was detected in samples at the end of-G-CSF administration (Figure 5.33 and table 5.9).

G-CSF administration significally reduced the capacity of EPC colony formation. However the reason for this decrease is as yet unknown. Further analysis is required before any conclusions can be drawn.

	PRE	PERI	POST
%CD45+	88.42	88.26	87.81
% CD14+	5.24	5.72	5.86
%CD34+	0.07	0.38	0.01
% CD133+	0.02	0.26	0.04
%CD29+	67.76	37.77	61.5
%CXCR4+	43.72	45.58	69.8
% VE-cadherin	4.78	5.59	5.83
% VEGFR2+	1.34	2.48	1.4
%CD34+CD45dim	44	0.2	25
%CD34+VEGFR2+	33.25	37.93	25
CD34+CD133+	30	84.95	87.5
%CD34+CD133+VEGFR2+	9.05	16.89	37.5
%CD14+CD34+	0.08	0.07	0
%CD14+VE-cadherin+	85.97	48	75.42
%CD14+VEGFR2+	3.63	17.67	0.42
%CD14+CD45+	98.98	99.67	99.92
%CD14+CD29+	99.05	96.35	99.19
%CD14+CXCR4+	91.2	90.8	99.11

Table 5.9 Comparison of the percentage of a panel of different surface markers pre, peri and post G-CSF administration.

Characterisation of non-adherent peripheral blood cells after 2-day primary culture by flow cytometry using a panel of antibodies specific for endothelial and haematopoietic cells pre, peri and post G-CSF administration (pre n=4, peri n=4 and post n=1).

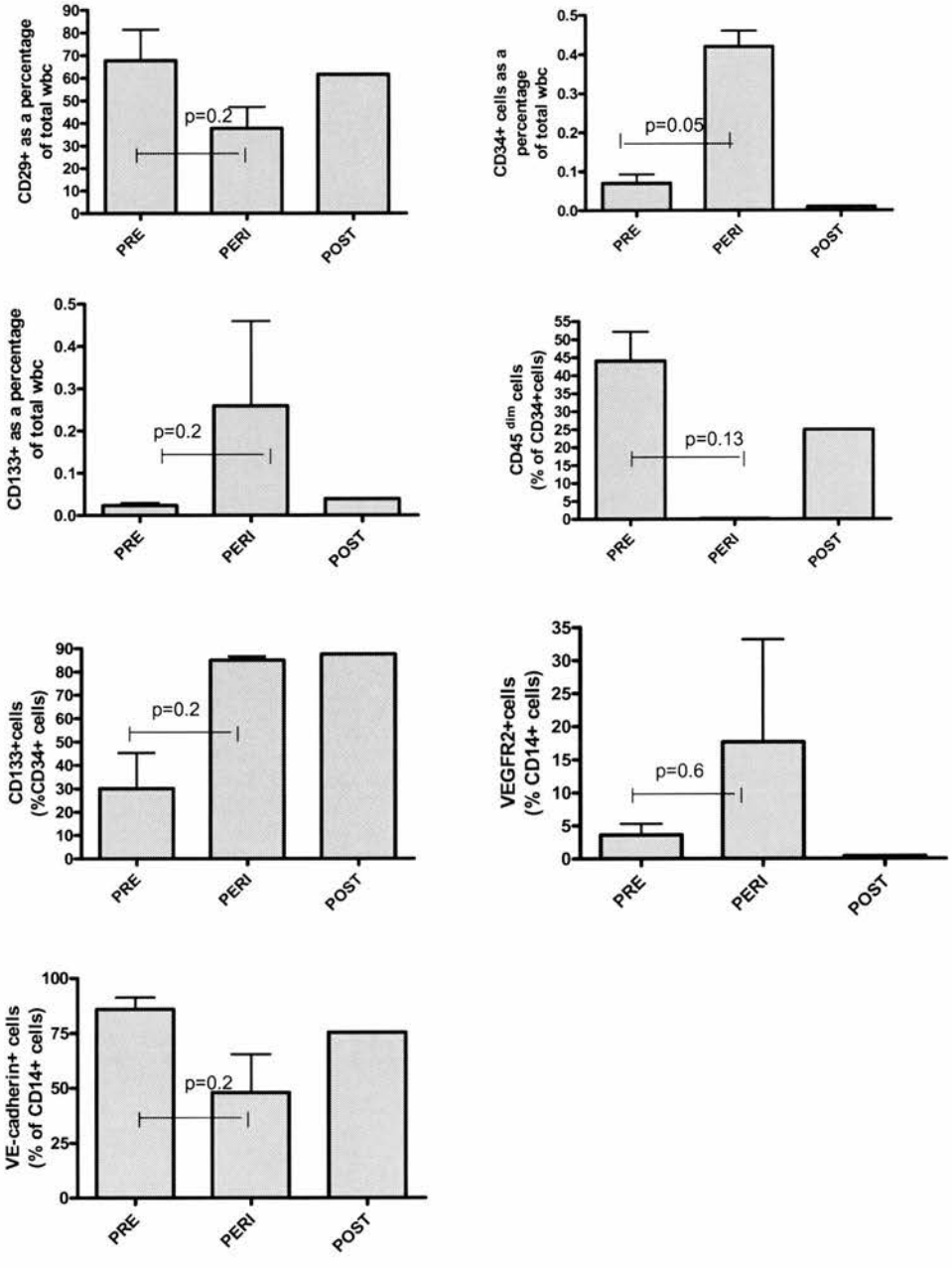


Figure 5.33 Changes in expression of surface markers by non-adherent peripheral blood cells after 2-day primary culture pre, peri and post G-CSF administration

Results show down-regulation, no change or up-regulation of a panel of antibodies specific for endothelial and haematopoietic cell markers in peripheral blood samples of healthy individuals before (pre), during (peri) and after (post) G-CSF administration.

5.2.3.11 Dose the addition of other growth factors influence CFU-EPC generation?

To examine the possibility that other growth factors could also affect CFU-EPC generation, peripheral blood MNC which remain non-adherent after the 2-day primary culture step on fibronectin-coated plates were treated with 100ng/ml of a range of different growth factors. Flt3-L, SDF-1, VEGF and GM-CSF enhanced CFU-EPC generation whereas treatment with 100ng/ml of G-CSF or TPO caused a reduction in CFU-EPC generation. Addition of 100ng/ml of bFGF and SCF did not change the number of CFU-EPC (Figure 5.34).

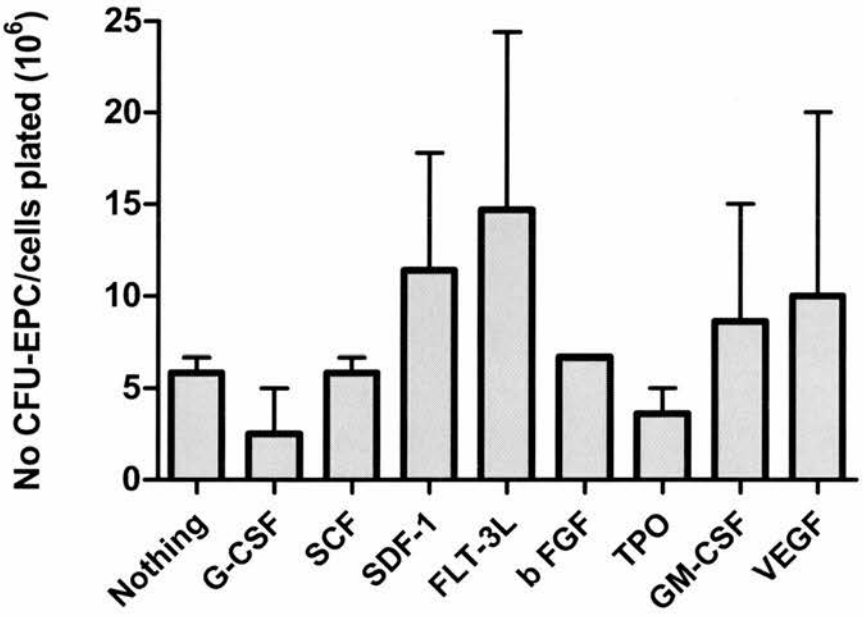


Figure 5.34 Effect of addition of different cytokines to the CFU-EPC assay

The addition of 100ng/ml of SCF, SDF-1, Flt-3L, G-CSF, TPO, GM-CSF, VEGF and bFGF was studied in terms of numbers of CFU-EPC. Results were expressed by the mean (SD) (n=2)

5.2.3.12 *In vitro* expansion of CD34$^+$ VEGFR2$^+$ (endothelial precursor phenotype) cells and maturation to the endothelial lineage

From previous studies on myeloid precursors (Chapter 3) we found that 100ng/ml SCF plus 10ng/ml Flt-3L was the optimal combination of cytokines for CD34$^+$ cell expansion *in vitro*, giving around 15-20 fold expansion in 14-day cultures (fold expansion being the number of cells after 14 days expansion divided by the number of CD34$^+$ cells before culture). Most of the cells after culture expressed CD34 brightly (see Chapter 3.3.3). In this study, we wanted to check if we could achieve (i) EPC expansion and (ii) endothelial differentiation. In order to expand/differentiate stem cells into the endothelial lineage we searched for specific growth factors with the capacity to drive these CD34$^+$-stem cells into the endothelial lineage.

5.2.3.12.1 Expansion of cell numbers

One of the first questions that we asked was if the presence of vascular endothelial growth factor (VEGF) would inhibit the expansion of CD34 expressing cells compared to our known optimal cytokine cocktail (SCF/Flt-3L) for CD34$^+$ expansion. VEGF is a potent growth and angiogenic cytokine. It stimulates proliferation and survival of endothelial cells, and promotes angiogenesis and vascular permeability (Shalaby *et al.*, 1995; Plate *et al.*,1992). Basic fibroblast growth factor (bFGF) is a potent angiogenic factor as well as a stimulator of blood vessel growth and an important player in wound healing (Slavin *et al.*,1995). Cells cultured with VEGF growth factor alone show minimal cell expansion. The presence of VEGF to the SCF/Flt-3L expansion cocktail did not increase the expansion neither did reduce it (33.01-fold expansion without VEGF versus 33.62 when VEGF was present) (Figure 5.35). 50ng/ml of VEGF instead of 100ng/ml VEGF also did not have any significant alteration of expansion numbers (Figure 5.36).

The addition of bFGF in the culture did not significally increase or reduce the expansion cell numbers (20.71-fold expansion with bFGF versus a 26.56 expansion without bFGF) (Figure 5.37). Similarly, the addition of 100ng/ml of the Sigma cytokine complex, an extract of bovine neural tissue containing growth promoting factors for vascular endothelial cells (Maciag *et al.*, 1982), to the previous culture conditions did not significally increase or reduce the expansion numbers (8.56 fold expansion with Sigma complex compared to 9.85 expansion with SCF/Flt3-L+VEGF alone)(Figure 5.38).

The presence of Heparin in endothelial cultures have been shown to optimise endothelial growth (Gargett *et al.*, 2000; Bagley, 2003). Thus,the addition of 10 Units/ml of heparin in the culture increased cell expansion though the result did not reach significance (11.04 with SCF/Flt3-L+VEGF+heparin) compared to without it (9.85 SCF/Flt3-L+VEGF) (Figure 5.38).

None of these growth factors gave significant improvements in the expansion numbers compared to SCF/FLT-3L/VEGF cytokine cocktail.

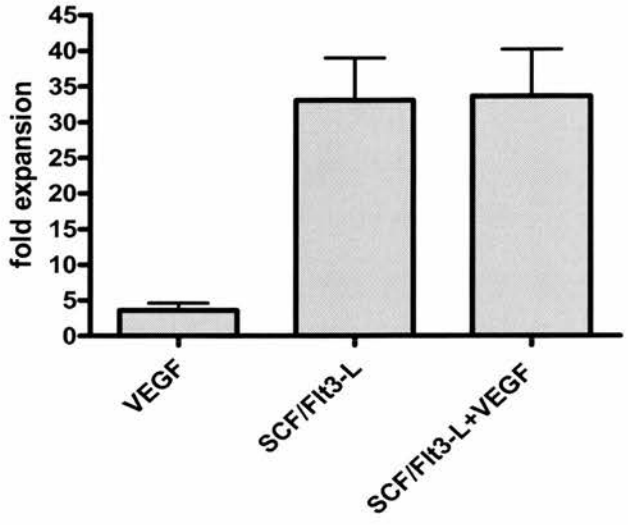


Figure 5.35 CD34⁺ cell expansion with or without the presence of VEGF growth factor
Fold expansion (number of cells after 14 days / number of cells at day 0 of culture) after cells culture with VEGF alone or with SCF/Flt3-L with or without the presence of VEGF. Data is presented as mean±SD (n=9 independent experiments).

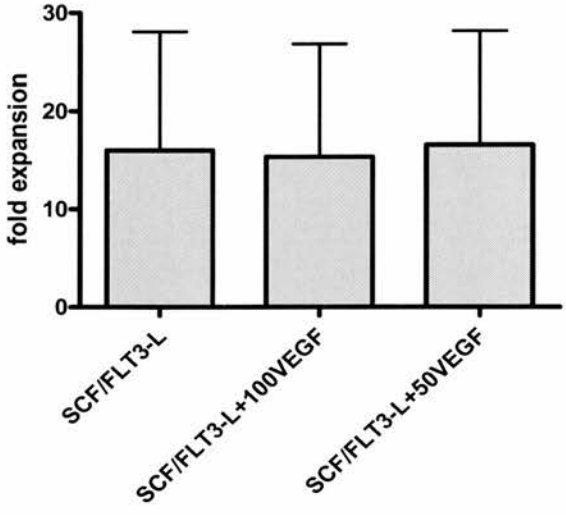


Figure 5.36 Effect of the addition of 50 or 100ng/ml of VEGF to the SCF/Flt3-L expansion cocktail.
Fold cell expansion of cells cultured with 50 or 100 ng/ml of VEGF growth factor with SCF/Flt3-L expansion cocktail. Data is presented as mean±SD (n=8 independent experiments).

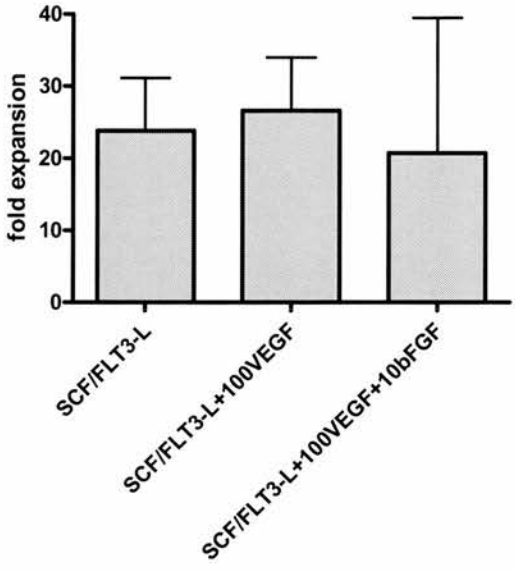


Figure 5.37 Effect of the addition of 10ng/ml of bFGF to the SCF/Flt3-L+50VEGF combination of cytokines

Fold cell expansion of cells cultured with 10ng/ml of bFGF with the SCF/Flt3-L+50 VEGF growth factors. Data is presented as mean±SD (n=4 independent experiments).

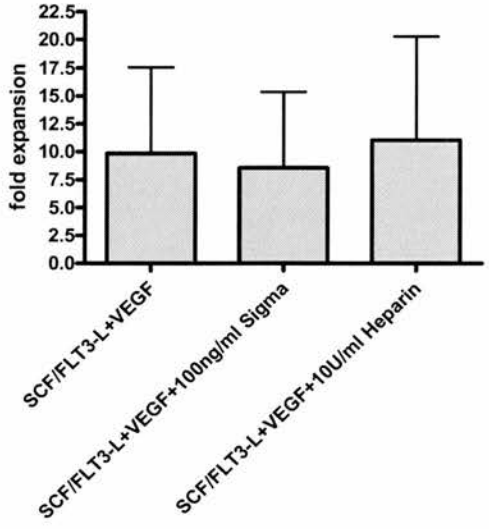


Figure 5.38 Effect of the addition of heparin or of an endothelial cytokine complex (Sigma, UK) to the SCF/Flt3-L+50VEGF combination of cytokines

Fold cell expansion of cells cultured with 10U/ml of heparin or with 100ng/ml of an endothelial cytokine complex created by Sigma together with SCF/Flt3-L+VEGF growth factors. Data is presented as mean±SD (n=4 independent experiments).

5.2.3.12.2 Endothelial cell differentiation

After 14 days of cytokine culture the percentage and the numbers of cells expressing VEGFR2 in the different cytokine combinations was calculated. The presence of VEGF growth factor induced an increase of the percent of VEGR2 and the total number of $VEGFR2^+$ cells/ml (Figure 5.39). Cells cultured with VEGF growth factor alone had a high percentage of $VEGFR2^+$ cells compared with the other combinations (45% $VEGFR2^+$ cells in the well of VEGF alone versus 37% SCF/FLT3-L+VEGF) (data not shown). However, the cell expansion was almost none ($0.03x10^6$ $VEGFR2^+$ cells/ml) (Figure 5.39). VEGF alone may protect the $VEGFR2^+$ cells but not the other cells, so they survive and the other cells die.

Cells cultured in the presence of SCF/Flt-3L+VEGF growth factors showed significant higher number of $VEGFR2^+$ cells ($0.18x10^6$ $VEGFR2^+$ cells/ml) compared to cells cultured with SCF/Flt-3L without VEGF ($0.11x10^6$ $VEGFR2^+$/ml) (p=0.007)(Figure 5.39) (being number of $VEGFR2^+$ cell/ml as the % of $VEGFR2^+$ cells x the number of $VEGFR2^+$ cells after 14 days of culture).

The use of 50ng/ml instead of 100ng/ml of VEGF did not significally matter in terms of the number of $VEGR2^+$ cells/ml after culture ($0.20x10^6$/ml using 100ng/ml versus $0.19x10^6$/ml using 50ng/ml of VEGF)(Figure 5.40). Therefore, we selected 100ng/ml SCF+ 10ng/ml Flt-3L + 50ng/ml VEGF as the endothelial expansion cocktail.

Figure 5.41 shows how we calculate the percentages based on the flow cytometer data.

Addition of 10ng/ml of bFGF to the base endothelial differentiation cocktail (100ng/ml SCF+ 10ng/ml Flt-3L + 50ng/ml VEGF) did not increase the numbers of $VEGR2^+$ cells after culture. ($0.17x10^6$ $VEGFR2^+$/ml with bFGF versus $0.18 x10^6$ with SCF/Flt3-L+VEGF) (data not shown).

100ng/ml of a Sigma Endothelial growth supplement (ECGS) that contain growth-promoting factors for vascular endothelial cells used in endothelial cell lines was added to the SCF/Flt3-L+VEGF cytokine combination as well of 10-100ng/ml of Heparin. Figure 5.42 shows that addition either heparin or the sigma cytokine complex did not increase significally the number of $VEGFR2^+$/ml after 14 days of culture ($0.39x10^6$ $VEGFR2^+$ cells/ml with heparin, $0.33x10^6$ $VEFGFR2^+$ cells/ml with the sigma cytokine complex versus $0.25x10^6$ $VEGFR2^+$ cells/ml with SCF/Flt-3L+VEGF).

On the other hand, high proportion of VEGFR2 expression but no expansion was observed when cells were cultured with the Sigma cytokine complex alone without the expansion SCF/Flt3-L cocktail (data not shown). Like cells cultured with VEGF

growth factor alone, Sigma cytokine complex might selectively protect VEGFR2$^+$ cells, while without (SCF/Flt-3L) growth factors the rest of the cells die.

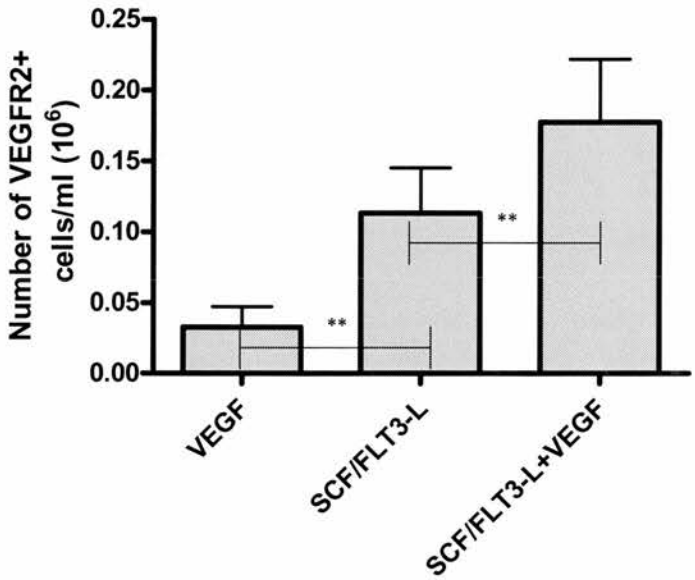


Figure 5.39 Endothelial cell differentiation

Number of CD34$^+$-derived VEGFR2$^+$ cells after ex *vivo* culture (% of VEGFR2$^+$ cells/ number of cells after 14 days of culture) after culture with VEGF alone ($0.03x10^6$ VEGFR2$^+$ cells/ml) or with SCF/Flt-3L with ($0.18x10^6$ VEGFR2$^+$ cells/ml) or without ($0.11x10^6$ VEGFR2$^+$ cells/ml) the presence of VEGF. Data is presented as mean±SD (n=8 independent experiments).

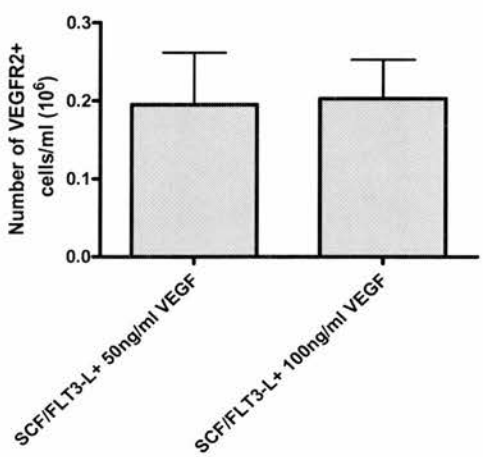


Figure 5.40 Endothelial cell numbers. Effect of the addition of 50 or 100ng/ml of VEGF to the SCF/Flt3-L growth factors.

Number of VEGFR2$^+$ cells after culture with 50 or 100 ng/ml VEGF to the SCF/Flt-3L combination of cytokines. Data is presented as mean±SD (n=5 independent experiments).

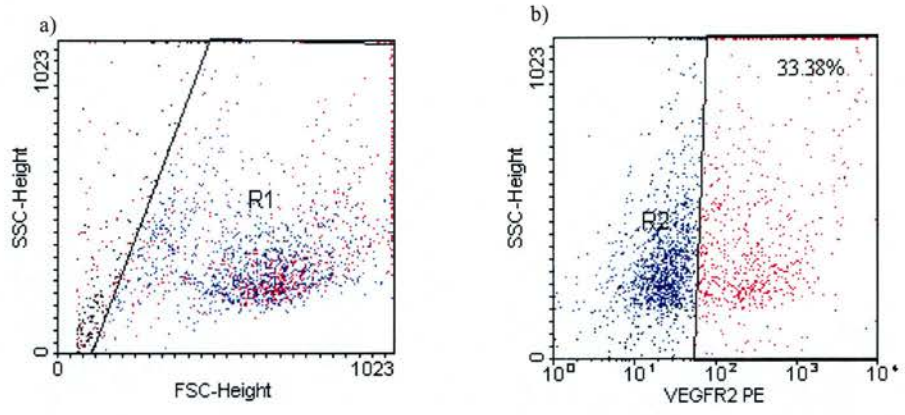


Figure 5.41 Flow cytometry analysis of the *ex vivo* endothelial differentiated cells.

Flow cytometry analysis with a) FSC/SSC and b) percentage of VEGFR2$^+$ gated on the FSC/SSC (magenta).

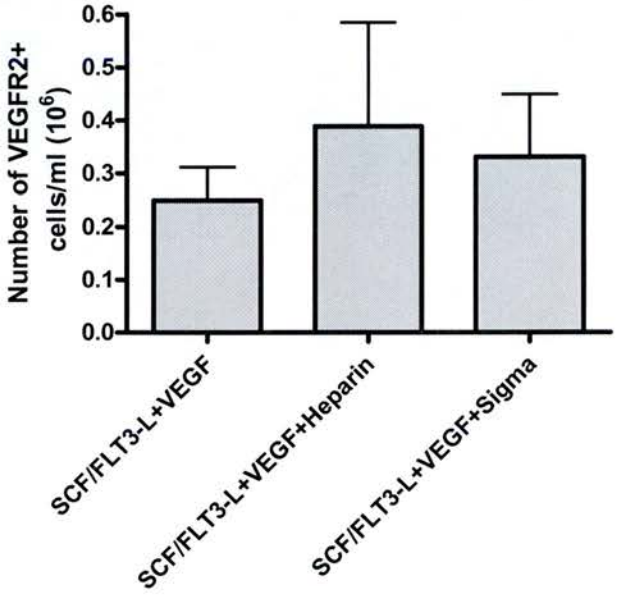


Figure 5.42 Endothelial cell numbers. Effect of the addition of heparin or of an endothelial cytokine complex (Sigma, UK) to the SCF/Flt3-L+50VEGF combination of cytokines

Number of VEGFR2$^+$ cells after culture with the presence of heparin or Sigma endothelial cytokine cocktail with SCF/Fl3-L+VEGF cytokine combination. Data is presented as mean±SD (n=4 independent experiments).

5.2.3.12.2.1 Clonetics endothelial rich media

No significant improvement in terms of cell expansion or endothelial cell differentiation was detected when the endothelial basal medium (EBM) (Clonetics, UK) plus added growth supplements (hydrocortisone, hFGF-B, VEGF, R3-IGF-1, Ascorbic acid, Heparin, hEGF, FBS, GA-1000) (Clonetics, UK) was used in comparison to the Iscove's Modified Dulbecco Media (IMDM) with 10% FCS (Biowhittaker,UK) 1% Antibiotic (penicillin/streptomycin) (10000Units/ml /10ug/ ml)respectively (Sigma, UK) (p=0.25) (Figure 5.43).

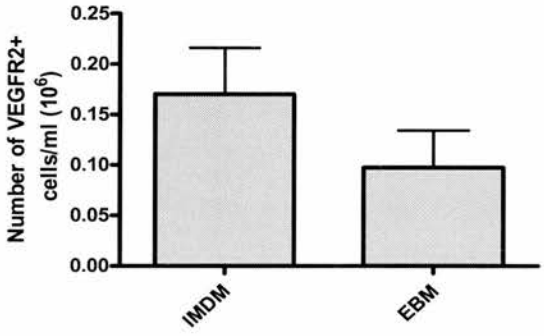


Figure 5.43 Endothelial cell numbers. Different culture mediums

Comparison of number of VEGFR2$^+$ cells/ml between IMDM (Iscove's, Modified Dulbecco's media) (Invitrogen, UK) with EBM (endothelial basal medium) (Clonetics, UK). Data is presented as mean±SD (n=3 independent experiments).

5.2.3.12.2.2.Culture on different substrates

No significant differences were detected in terms of number of VEGFR2$^+$ cells/ml when cells were plated on fibronectin pre-coated wells or on gelatin pre-coated wells compared to un-coated wells (Figure 5.44).

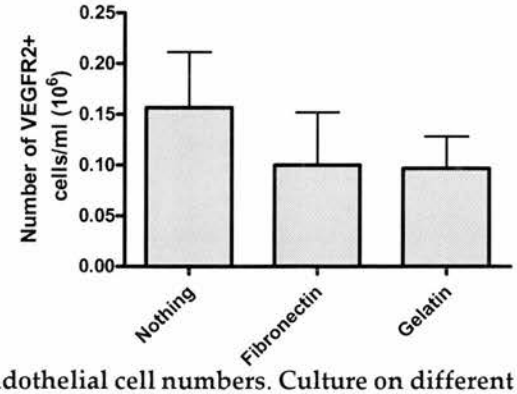


Figure 5.44 Endothelial cell numbers. Culture on different substrates

Number of VEGFR2$^+$ cells/ml cultured in none, fibronectin or gelatin substrates. Data is presented as mean±SD (n=3 independent experiments).

5.2.3.13 CD34⁺ cells cultured with VEGF and subsequent CFU-EPC potential.

Expansion of CD34⁺ cells from cord blood, G-CSF mobilised peripheral blood, peripheral blood or bone marrow in the presence or absence of VEGF (SCF/Flt-3L±VEGF) prior to CFU-EPC assay showed that both conditions generate spindle-shaped cells but few endothelial colonies which were small (Figure 5.45). However, this was more frequent following exposure to VEGF, which also resulted in some cases in the formation of structures. Comparing different sources, cord blood gave slightly higher numbers of spindle-shaped cells and CFU-EPC than did mobilised peripheral blood (data not shown). Spindle-shaped cells were also shown to be positive for DiI-Ac-LDL staining.

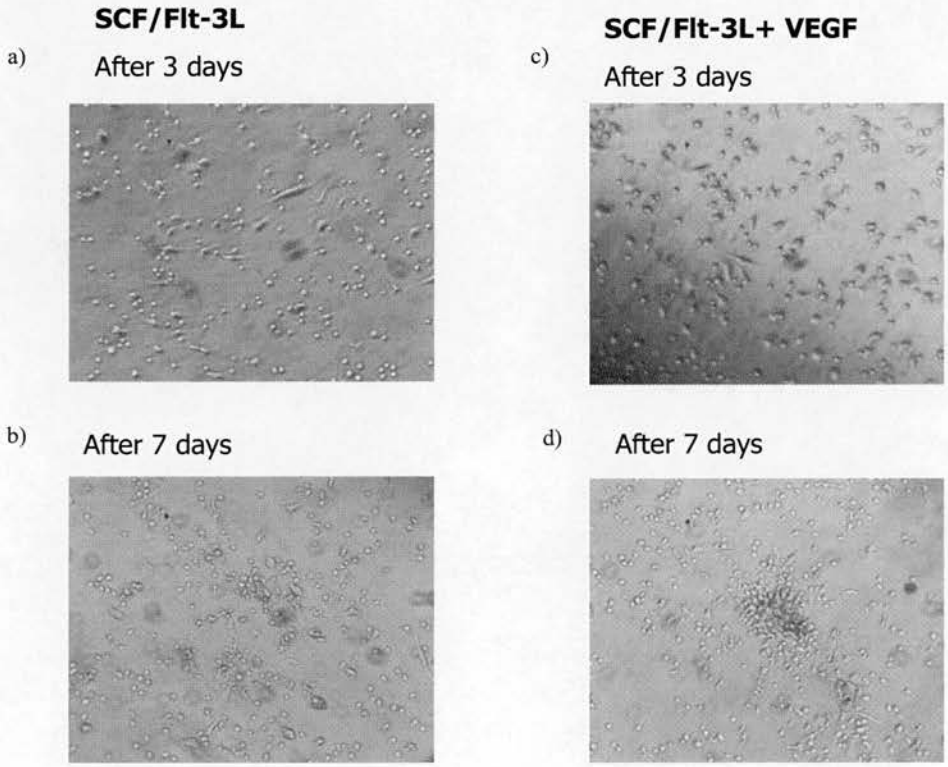


Figure 5.45 Endothelial progenitor cell colony assay (CFU-EPC) in *ex vivo* endothelial differentiated cells

CD34⁺ cells differentiated after 14 days culture with SCF/Flt-3L alone (a,b)or with the addition of VEGF (c,d) were then assessed for the CFU-EPC potential. Spindle-shaped cell morphology and endothelial colonies were observed a,c) after 3 days , b,d) after 7 days culture. Blood source: Cord blood

5.2.3.14 CD34$^+$ cells cultured with GM-CSF and subsequent CFU-EPC potential

CD34$^+$ cells were enriched and expanded with SCF/Flt-3L in the presence of GM-CSF for 14 days prior to CFU-EPC assay. GM-CSF is a haematopoietic growth factor that stimulates the development of neutrophils and macrophages and enhances the functional activity of the mature end-cells (Jones, 1996). At 7 days, expanded cells from all sources tested showed a rounded morphology and a high expansion potential as observed in culture. After an extended culture period in CFU-EPC assay (14-25 days) some spindle-shaped cells and CFU-EPC colonies appeared (Figure 5.46). These occurred earlier in cells derived from nPB and BM than was the case for CB and MB sources. Cultured BM derived CD34$^+$ cells showed formation of cord structures (Figure 5.46 and 5.47).

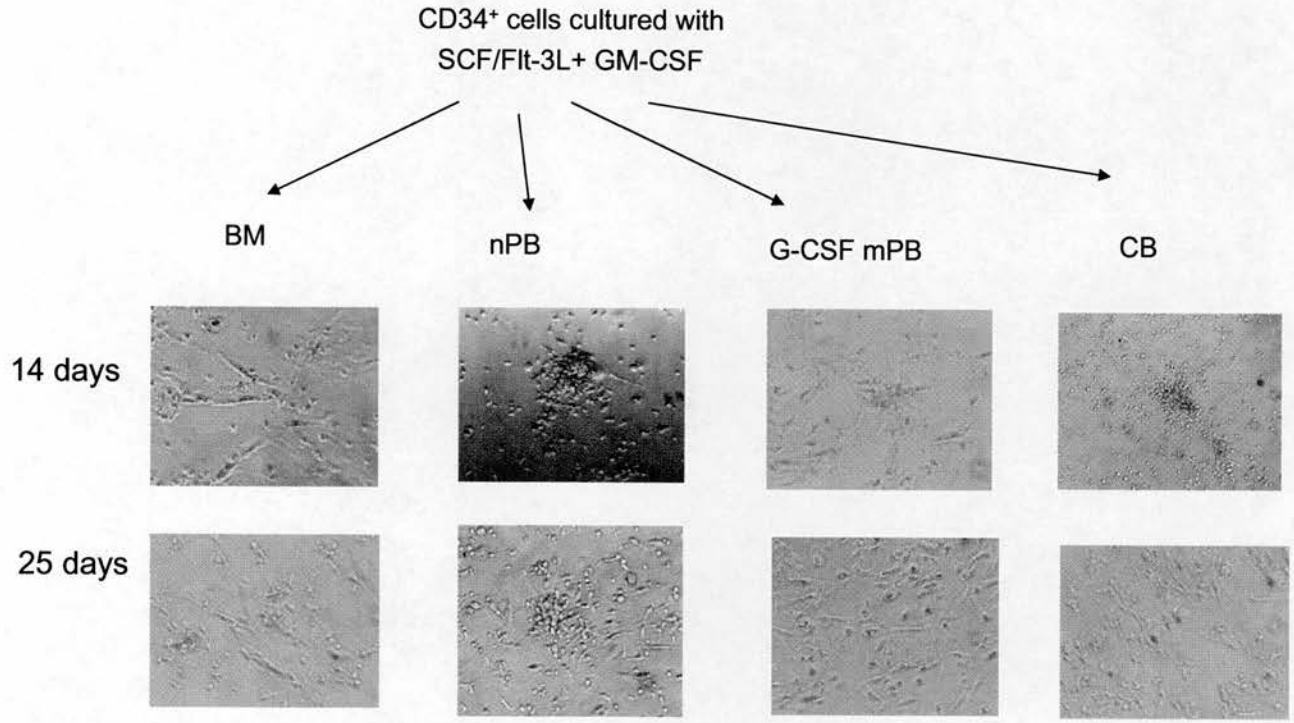


Figure 5.46 CD34⁺ cells cultured with SCF/Flt3-L+GM-CSF and subsequent CFU-EPC potential

CD34⁺ cells from different sources (bone marrow, normal peripheral blood, mobilised peripheral blood and cord blood) differentiated after 14 days with SCF/Flt3-L+GM-CSF. Recovered cells were then assessed for CD14⁺ expression and CFU-EPC potential. Spindle-shaped cell morphology and endothelial colonies were observed after 14-25 days in complete endothelial culture medium (CECM).

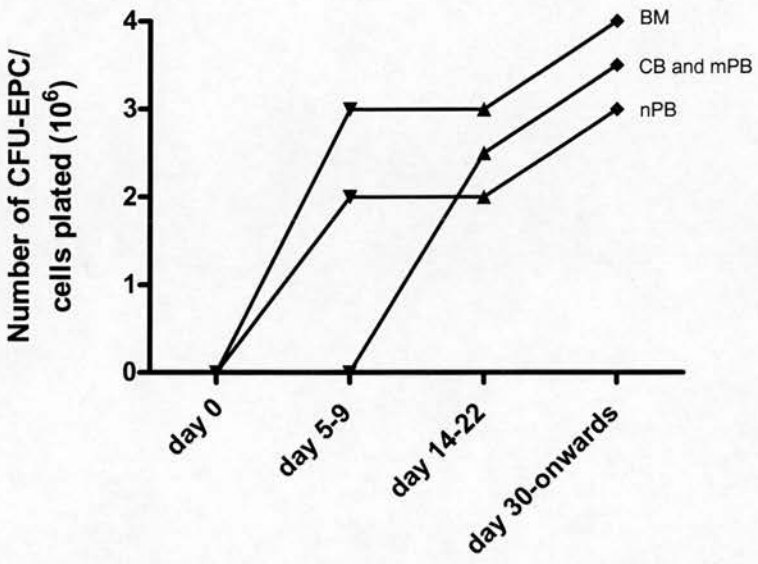


Figure 5.47 Formation of CFU-EPC over different time points
Differential speed of endothelial colony formation in the different sources used (normal peripheral blood, bone marrow, cord blood and mobilised peripheral blood) of the CD34-derived cells after SCF/Flt3-L/GM-CSF culture

5.2.3.15 MNCs differentiated to dendritic cells

Peripheral blood $CD14^+$ cells produce both CFU-EPCs and dendritic cells. $CD14^+$ cells differentiated to dendritic cells in the presence of GM-CSF and IL-4 (See 2.5.3). When the CFU-EPC was assessed for such cells no colonies were detected (Figure 5.48) and the cells had typical dendritic cell morphology and phenotype as assessed by flow cytometry. Cells high in forward and side scatter were brightly positive for CD209 (DC-SIGN), CD11c, CD86, CD58 and HLA Class I and Class II markers (Figure 5.49).

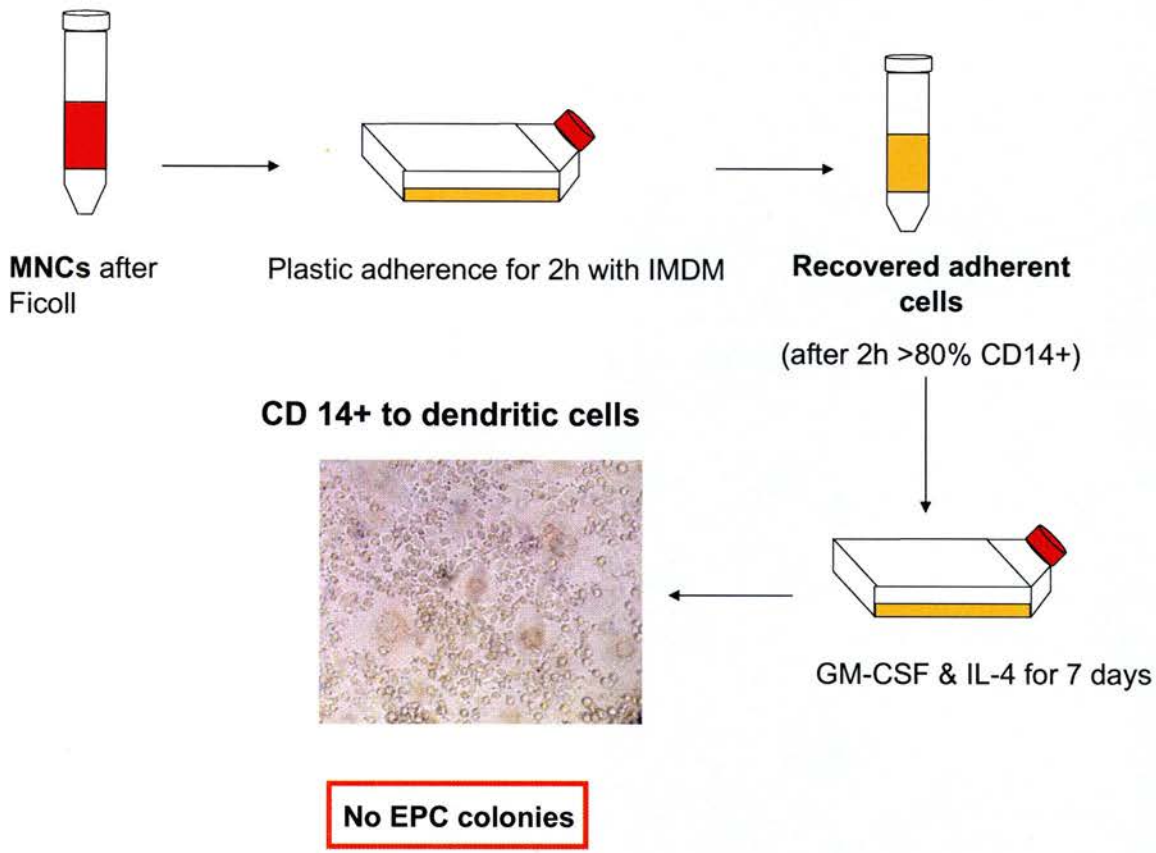


Figure 5.48 Schematic representation of dendritic cell differentiation from CD14⁺ cells
Mononuclear cells were adhered for 2h in a plastic flask, recovered and cultured in IMDM with the presence of GM-CSF and IL-4 growth factors. After 7 days recovered cells were assessed for CFU-EPC potential.

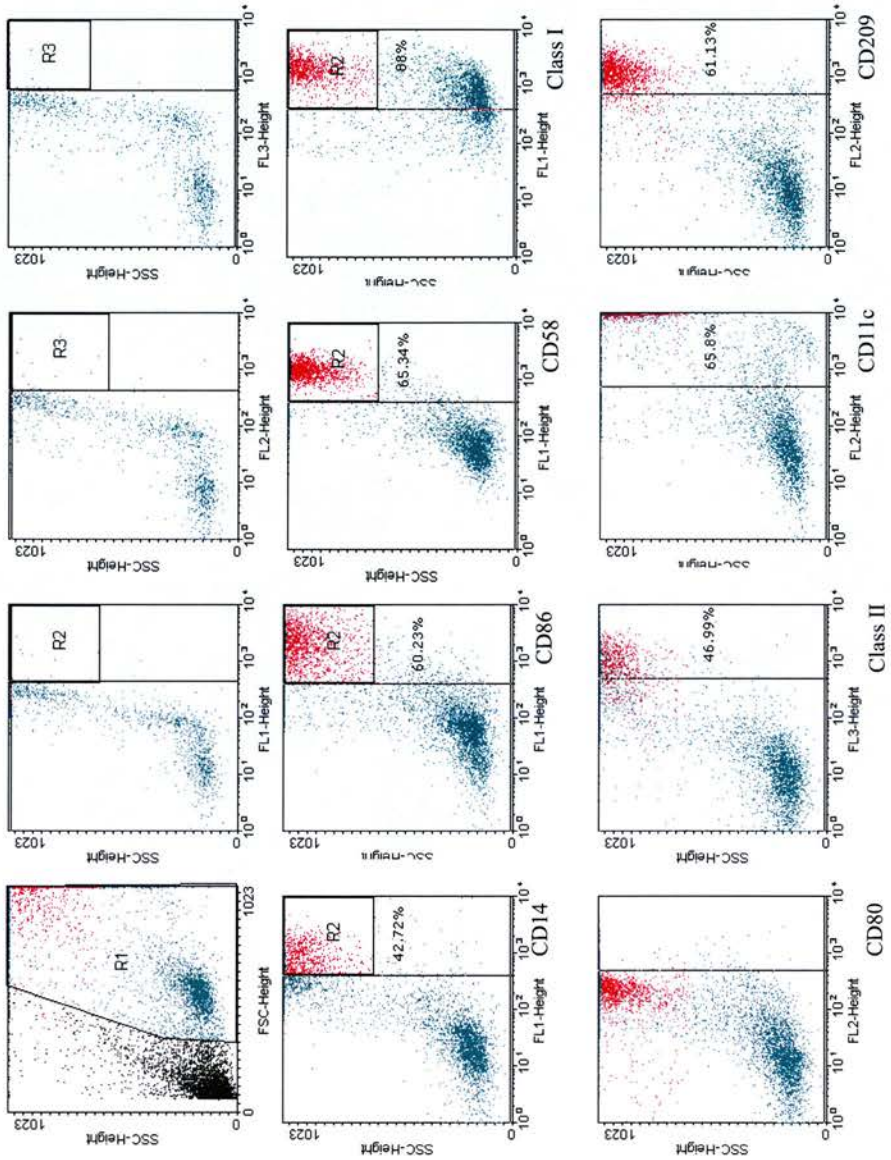


Figure 5.49 Dendritic cell characterisation

Flow cytometry analysis was used to phenotype 2h adherent cells recovered after 7 days in GM-CSF and IL-4 growth factors. Expression of a panel of dendritic markers showed that these cells were positive for the most common dendritic markers. First line show from left to right; FCS/SSC, FL1-H (FITC), FL2-H (PE) and FL3-H (PercP) negative controls.

5.2.3.16 Matrigel endothelial tube formation assay.

Mature endothelial cell lines have been shown to generate tubule structures in matrigel cultures. However, in this study tubule formation was not seen either using *ex vivo* cultured endothelial cells nor peripheral blood derived sub-populations including $CD133^+$, $CD34^+$, 2h plastic adherent, 2h plastic non-adherent, $CD14^+$ or $CD14^-$ populations (data not shown).

5.2.3.17 Is there any relationship between adherent CFU-EPC CD14+ cells and Mesenchymal stem cells (MSC)?

(Study in collaboration with Kay Samuel, SNBTS)

MNCs were plated into a flask for 4 days in Mesencult media (Stem Cell Technologies, UK). Adherent cells after culture were considered putative MSCs able to differentiate into bone cells (Kay Samuel, unpublished)

These putative mesenchymal stem cells (MSCs) were also plated in 24-well fibronectin-coated plate in CECM to assess their CFU-EPC potential. After 5 days in culture putative MSCs formed 35 CFU-EPC/10^6 cells plated.

In parallel, non-adherent cells also harvested after 2 days on fibronectin as for the primary step of the CFU-EPC assay described by Hill *et al.*, (2003) generated 10 CFU-EPC/10^6 cells plated, but also after various expansion passages were positive for bone cell markers (Figure 5.50). Further, different enriched cell populations such as $CD14^+$, $CD14^-$, $CD34^-$, $CD34^+$, $CD133^+$, $CD133^-$, were also capable to differentiate to bone cells (data not shown).

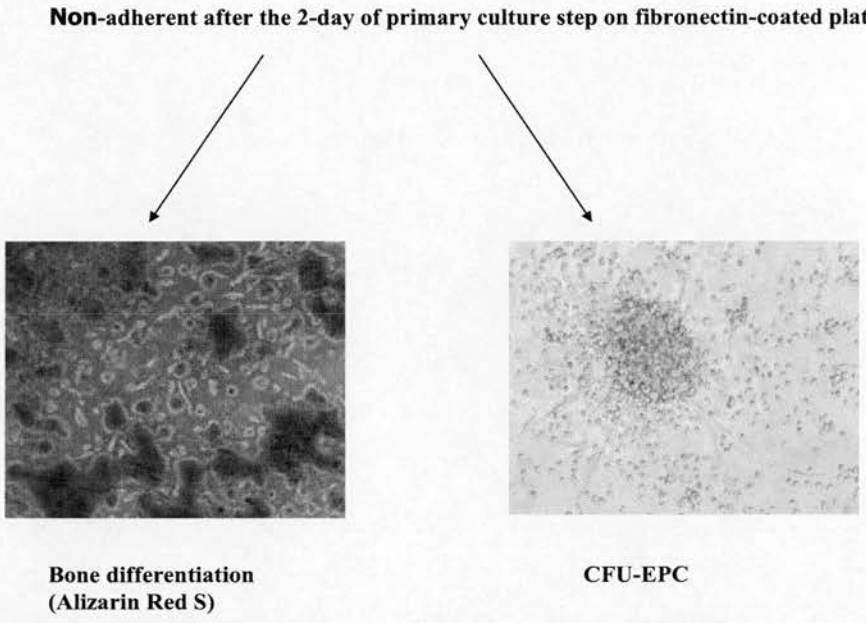


Figure 5.50 Endothelial and osteogenic differentiation potential of the non-adherent 2-day fibronectin cells.

Double differentiation potential of non-adherent cells harvested after 2 days on fibronectin as for the primary step of the CFU-EPC a) osteogenic cell differentiation assessed by Alizarin Red S staining and b) CFU-EPC potential (Hill *et al.*, 2003).

5.2.3.18 Characterisation and CFU-EPC capacity assessment through selective adhesion time points

Peripheral blood MNCs were allowed to adhere to plastic for 2, 24, 48, 72 or 120 hours in 25cm^2 tissue culture flasks in IMDM media. Cells remaining non-adherent were discarded.

There was a gradual decrease in terms of CFU-EPC potential and endothelial cellurarity thought the time points. 2h-adherent cells were the cell population which generated the highest number of endothelial progenitor colonies whereas recovered adherent cells after 72 hours in culture had no CFU-EPC potential (Figure 5.51).

Surface markers were analysed by flow cytometry and compared between the adherent cells at the different time points.

VEGFR2, VE-cadherin, CD14, CD16, CD146, CD3 and CXCR4 positive expression increased through the time points with a peak at 48h. CD45 and CD29 expression decreased with longer adherent culture times and CD34 and CD133 expression remained unchanged. CD14$^+$ cell subsets were also analysed. CD14$^+$ cells co-expressing CD34 or VE-cadherin increased through the longer time points with a

peak at 48h. $CD14^+$ cells co-expressing CD45 decreased though the time points and no-significant change was detected in other $CD14^+$ cell subsets such as $CD14^+CD29^+$or $CD14^+CXCR4^+$ (Table 5.10).

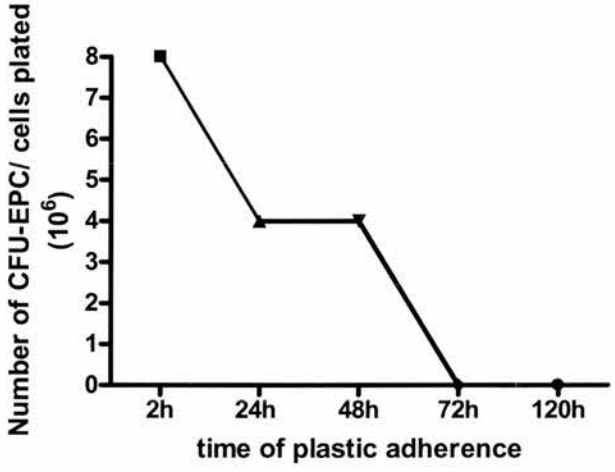


Figure 5.51 Characterisation and CFU-EPC potential through selective adhesion time points

Gradual decrease in terms of CFU-EPC numbers thought 2, 24, 48, 72 or 120 hours adherence in $25cm^2$ uncoated tissue culture flasks.

041005 BC adherent cells	2h	24h	48h	72h	120h
%CD45+	93.43	85.62	68	51.11	50
%CD34+	0.08	0.06	0	0	0.1
%CD133+	0.05	0.05	0	0	0.05
%CD34+CD45-	12.12	25	0	0	
%CD34+VEGFR2+	54.55	62.5	0	0	
% VEGFR2+	2.42	22	67.69	29.63	20.18
% VE-CAD+	0.27	40.17	27.66	?	11.77
% CD3+	5.11	0.31	4.36	47.15	37.46
% CEC	3.5	3.22	55.31	15.56	25.48
% CXCR4+	41	65.75	66	39.85	33.98
% CD29+	67.52	?	58.43	25.81	43
% CD16+	0.71	32.77	59.41	20.71	12
% CD14+	26.42	65.61	58	12.42	12.28
% CD14+CD45+	100	99.19	94.92	80	85
%CD14+CD34+	0.3	0.58	1.69	0	0
%CD14+VEGFR2+	97.48	?	97.46	78	87
%CD14+VE-CAD+	9.57	25	95.12	87	80
%CD14+CD29+	99	98.7	95	92.5	97
%CD14+CXCR4+	96.12	79.62	97.56	90	65

Table 5.10 Phenotype analysis by proportions in cells after different adherence time points

Surface expression of a panel of markers specific for endothelial or haematopoietic cells was analysed by flow cytometry and compared between adherent cells at the different time points.

5.3 Discussion

5.3.1 Preliminary investigations

5.3.1.1 Use of endothelial cell lines as indicators of mature EC phenotype and function

Endothelial cell lines expressing markers of mature endothelial cells and having mature endothelial cell function were used in this study as controls for the assays used to identify endothelial cells (EC) and endothelial progenitor cells (EPC). Both HUVEC and the immortalised hybridoma endothelial EA.hy926 cell line showed characteristic endothelial cell properties based on expression of markers detected by flow cytometry, immunofluorescence and endothelial tube formation capacity. However, EA.hy926 cells were much easier to culture, grew more quickly and had indefinite expansion potential. Therefore, the EA.hy926 cell line was used in preference to HUVECs.

The functional capacity difference between progenitor and mature endothelial cells is currently unknown. The EA.hy926 cells appear to act like mature endothelial cells and were capable of endothelial tube formation in Matrigel, but were not able to form endothelial progenitor colonies in Hill's assay. Other studies have shown that differentiated mature endothelial cells do not improve neovascularisation (Kalka *et al.* a), 2000; Hur *et al.*, 2004; Kocher *et al.*, 2001). This suggests that an as yet undefined functional characteristic of EPC compared to EC is essential for vascular repair and regeneration following damage of endothelium leading to ischaemia (Urbich and Dimmeler, 2004).

5.3.1.2 Platelet-leucocyte aggregates preclude use of antibody to markers shared by platelets and endothelial cells.

A number of reports employ anti-Von Willebrand factor (vWf) antibody to determine vWf expression as a marker of EC maturity. However, flow cytometry results showed unexpected high expression of vWf by different freshly isolated leucocyte subpopulations because of platelets adhered to their surface. Our results indicate that EDTA anticoagulant has a lower effect on this platelet-leucocyte aggregation effect and it was prioritised for the collection of our blood samples. Moreover EDTA/PBS was used as a washing solution whenever samples were taken with heparin anticoagulant. EDTA/PBS washing solution presented a reduction in platelet-leucocyte aggregation compared to other washing solutions used. Even though

platelet-leucocyte aggregation was reduced high amounts of vWf positive cells were still detected by flow cytometry. Thus, we could not assure that all vWf seen were indeed mature endothelial cells. vWf was not used as endothelial marker in our panel of endothelial antibodies

5.3.1.3 Unexpected expression of VEGFR2 and VE-cadherin on PBMNC populations

Monoclonal anti-humanVEGFR2 (KDR) (R&D systems) is thought to be specifically a marker of endothelial cells. When tested in mature endothelial cell lines the expression is specific. However, analysis of whole blood showed that other cells, in particular granulocytes and monocytes expressed VEGFR2. This was not seen in all samples tested. The incidence of VEGFR2 expression changed in the different sources and in different samples collected at different time points following a clinical procedure. Similarly, cells other than endothelial cell populations in all sources were found to express VE-cadherin. VE-cadherin expression was mostly associated with cells having scatter characteristics of monocytes (mainly in mobilised peripheral blood samples) and lymphocytes (mainly in cord blood samples). The cause of this VEGFR2 and VE-cadherin unexpected expression by these cells is unknown. However, these markers specifically stained cultured mature endothelial cell lines and cells after *ex vivo* culture. $CD34^+$ and/or $CD133^+$ cells did not experience this unexpected expression Therefore, VEGFR2 and VE-cadherin markers were still used as endothelial markers in these situations.

5.3.2 Putative EPC phenotypes in different HSC sources

Identification and enumeration of angiogenic stem cells present in the adult remains difficult and non-standardised. No single marker, truly specific for endothelial cells has been identified; therefore it is currently necessary to use combinations of markers to best identify mature circulating endothelial cells (CEC) and immature endothelial progenitor cells (EPC). We have investigated the presence of the currently proposed EPC phenotypes in the most common and practicable potential EPC sources. All sources except normal peripheral blood (nPB) had substantial numbers of $CD34^+$ and $CD133^+$ cells, with mobilised peripheral blood being the source that had significantly higher total numbers of $CD34^+$ and $CD133^+$ by comparison to the other sources. We found that mobilised peripheral blood also had higher proportions

of $CD34^+$ cells which co-expressed $CD133^+$. This result agrees with findings of de Wynter *et al.*, (1998) who showed that 75% of $CD34^+$ from post-G-CSF aphaeresis samples were $CD133^+$ compared with 50% in cord blood and 35% in bone marrow. Thus in mobilised peripheral blood there is a strong correlation between expression of CD34 and CD133 markers.

It is acknowledged that CD133 is expressed ontogenetically before CD34 and may therefore be an antigen marking more primitive cells. Thus a CD34-negative$CD133^+$ cell may represent a more multipotent immature cell population. Our results show (table 5.4) that bone marrow, cord blood and normal peripheral blood had higher proportions of CD34-negative$CD133^+$ cells than mobilised peripheral blood sources. This could suggest that normal peripheral blood, bone marrow and then cord blood are sources that contain stem/progenitor cell populations with a higher proportion of more immature cells than mobilised peripheral blood. Bone marrow, normal peripheral blood and cord blood also had higher proportions of $CD34^+CD133^-$ cells, which by this reasoning can be considered a more mature stem cell population, compared to mobilised peripheral blood sources.

The pathway of HSC maturation is not fully defined, and it is possible that expression of CD34 and CD133 may be continuously up and down-regulated. Indeed Quesenberry *et al.*, (2002) have suggested that stem cells are continuously altering their phenotype and that these alterations are reversible. Thus, many described cell phenotypes could represent a single cell in different functional states. Moreover, Lemoli *et al.*, (2005) suggested that rather than a hierarchical transition from stem cells to progenitor cells, fluctuation continues to exist in which stem cells adjust their phenotype depending on the kinetic state of the cell; activated by their genetic program or microenvironmental stimuli.

Overall, the characteristic expression of the CD34 and CD133 markers in mobilised peripheral blood samples is completely different from that seen in normal peripheral blood, bone marrow and cord blood and its clinical relevance is unknown. It may be that G-CSF administration drives stem cells to a different kinetic state altering their HSC phenotype and may be priorising myeloid HSC differentiation. In contrast, phenotype expression of the proportions of CD34 and CD133 co-expression in non-mobilised peripheral blood follows a closer pattern to that seen in bone marrow than to mobilised peripheral blood samples.

Expression of CD45 is used to define cells of the haematopoietic lineage. $CD34^+$ cells with no or dim CD45 expression could represent a more primitive stage of HSC differentiation, which may generate $CD34^+CD45^+$ cells. These more primitive cells could give rise to cells of the haematopoietic lineage and also to non-haematopoietic

cells such as EPCs. Conversely, it is possible that $CD34^{+}CD45^{dim}$ cells represent a more mature $CD34^{+}$ cell differentiating into an endothelial cell type having lost CD45, since CD34 is also expressed on mature EC. In either case, our results show that the proportion of $CD34^{+}CD45^{dim}$ cells is much lower in mobilised peripheral blood samples, from both patients and allogeneic healthy donors, than is detected in normal peripheral blood, cord blood or bone marrow.

Again it is the mobilised blood samples that presented a very different characteristic expression of $CD34^{+}CD45^{dim}$ cells compared to the other sources tested, and normal peripheral blood $CD34^{+}$ cells had much similar proportions of $CD45^{dim}$ cells to bone marrow and cord blood despite their having much smaller numbers of $CD34^{+}$ cells in total. This may be due to the stimulatory effect of G-CSF administration. Again G-CSF could selectively drive HSC myeloid differentiation which are CD45 bright cells. In general, results for mobilised peripheral blood for both patients and donors are similar, and probably indicate that the findings are not a reflection of underlying differences in the patients whose blood was mobilised relating to any preceding therapy for their leukaemia, since normally healthy donors PBHSC show similar results following G-CSF administration. The significance and clinical relevance of these differences between mobilised peripheral blood HSC and non-mobilised peripheral blood and other HSC sources has not been addressed, though it does raise the possibility that some loss of stem cell multipotency has occurred.

Endothelial progenitor cells (EPC) are defined by some (Pelosi *et al.*, 2002; Botta *et al.*, 2004; Vasa *et al.*, 2001; Quirici *et al.*, 2001; Hristov *et al.* b), 2003)(see table 5.1) as cells that share haematopoietic stem cell markers such as CD34 and/or CD133 and also express a more specific endothelial marker such as VEGFR2. In our results cord blood is the haematopoietic stem cell-rich source that had the highest proportion of $VEGFR2^{+}$ cells as a fraction of the gated $CD34^{+}$ population, followed by bone marrow and then mobilised peripheral blood. However, normal peripheral blood shows almost three times the proportion of $CD34^{+}$ cells which are $VEGFR2^{+}$ compared to cord blood. Conversely, due to the low $CD34^{+}$ cell numbers in normal peripheral blood the absolute numbers of $CD34^{+}VEGFR2^{+}$ cells/ml are much lower in nPB than in BM, CB and mPB sources. Most of the $CD34^{+}VEGFR2^{+}$ cells in cord blood and normal peripheral blood do not express CD133.

The presence of EPCs as defined by other groups was also investigated, such as $CD133^{+}VEGFR2^{+}$ cells. Normal peripheral blood had the highest proportion of $CD133^{+}VEGFR2^{+}$ cells compared to the other sources and bone marrow had the lowest. For $CD133^{+}$ cells co-expressing CD34 and VEGFR2, though this population was almost below our limits of detection, mobilised peripheral blood and normal

peripheral blood had the highest number compared to bone marrow or cord blood. There was no significant difference between the HSC-rich sources analysed when the $CD34^-VEGFR2^+$ sub-population of $CD133^+$ cells was compared. However normal peripheral blood $CD133^+$ cells had a 3-fold higher proportion of these cells than did bone marrow.

In normal peripheral blood, proportions of the co-expression combinations of CD34, CD133 and VEGFR2 were much higher than in bone marrow, cord blood or mobilised peripheral blood. There was a lower percentage of $CD34^+$ cells or $CD133^+$ cells which did not co-express VEGFR2 compared to the other sources. However few of them stained all together. Higher proportions of $CD34^+VEGFR2^+$ cells were negative for $CD133^+$ compared to the other sources and equally higher proportions of $CD133^+VEGFR2^+$ were negative for $CD34^+$ cells. Even though peripheral blood is the source with higher proportions of these putative EPC phenotype cells it also has the lowest number of $CD34^+$ and $CD133^+$ cells. Therefore, in total numbers these phenotypes were similar or lower than the other studied sources.

Subpopulations of cells showing different combinations of expression and co-expression of CD34, CD133 and VEGFR2, which may represent EPC phenotypes according to different publications, differ widely in proportion in the sources tested. It is very difficult to interpret and compare results between published studies because each group uses an individual EPC definition based on their experimental and/or clinical preferences. CD34 and CD133 are both haematopoietic markers, but these populations are only partially overlapping. Only about 53% in cord blood and even a lower proportion in bone marrow and normal peripheral blood of the $CD34^+$ cells share the expression of the CD133 marker (see 5.2.2.4). Similarly, VEGFR2 antigen is expressed by subpopulations of each of these fractions and only a very low percentage of these cells co-express all three markers together (see 5.2.2.9). Therefore, there is little chance that all published definitions of EPC refer to the same cell population. The combination of the expression of these three markers could reflect an hierarchy of EPC differentiation or cells in different kinetic states. Thus many of these phenotypes may partly define endothelial progenitors of different ontogenic status, which would explain why different groups using differently defined cell subpopulations achieved similar results clinically. However, this cannot be determined without the availability of individual clonogenic assays for each EPC phenotype (Ingram *et al.*, 2004). Ingram *et al.*, (2004) described a single cell approach that identifies a novel hierarchy of endopoiesis (analogue to haematopoiesis) based on the single EPC clonogenic and proliferate potential. They proposed a terminology similar to that used for defining haematopoietic cell progenitors. High proliferative

potential endothelial colony-forming cell (HPP-ECFCs) give rise to macroscopic colonies that form secondary and tertiary colonies on replating. These cells can give rise to all subsequent stages of endothelial progenitors. The next endothelial stage is a cell called LPP-ECFC that form colonies but they do not form secondary colonies on replating. They can give rise to endothelial cell clusters that do not replate into colonies or clusters and differentiate to a more mature endothelial cell.

The determination of which phenotype more accurately defines an EPC is essential for further applications. With a better understanding of EPC origin and a more precise EPC phenotype definition, more consensus between results, better and purer EPC isolations and fewer opportunities for misinterpretation will follow.

The $CD34^+VEGFR2^+$ phenotype is currently the most widely used to define endothelial progenitor cells (Pelosi *et al.*, 2002; Botta *et al.*, 2004; Vasa *et al.* 2001), but $CD133^+VEGFR2^+$ (Gill *et al.*, 2001) and $CD34^+CD133^+VEGFR2^+$ (Quirici *et al.*, 2001; Vasa *et al.*, 2001; Peichev *et al.*, 2000) are also popular, (also summarised in table 5.1).

To elucidate which source has the greatest potential to generate EPC, an *in vitro* functional EPC colony assay (CFU-EPC) was used. Measurement of CFU-EPC, which is able to identify very low frequencies of such cells which would be difficult to quantify by flow cytometry, should provide a sensitive indicator of the capacity of circulating mononuclear cells to form endothelial cells. Unexpectedly, cord blood which was found to have the highest proportion of $CD34^+VEGFR2^+$ cells between the haematopoietic stem cell-rich sources, generated one of the lowest CFU-EPC numbers. Bone marrow was identified as the best source of CFU-EPC within the HSC sources, as compared to both cord blood and mobilised peripheral blood. Mobilised blood both from patients and from healthy donors was almost unable to generate any CFU-EPC. However, normal (non-mobilised) peripheral blood, though it had the lowest number by far of $CD34^+$ and/or $CD133^+$ cells, was the source with the best potential to generate CFU-EPC colonies, more than double that seen for bone marrow.

We found no association between the number of cells expressing CD34 and CD133 alone or in combination with VEGF and the number of CFU-EPCs generated. Therefore, cells identified as EPCs using current phenotype definitions do not appear necessarily to be the population of cells as defined by the *in vitro* CFU-EPC functional assay. Though peripheral blood had high proportions of cells co-expressing CD34, CD133 and or VEGFR2, the total cell numbers were lower than or similar to the other sources studied. Yet normal peripheral blood had the highest numbers of EPC colony forming cells. Therefore, it is unlikely that $CD34^+$ cells or

$CD133^+$ cells identify circulating EPC in adult peripheral blood.
It is possible that the phenotype definitions of EPC currently in use are not definitive, that the *in vitro* CFU-EPC does not detect true EPCs, or that the two methods used detect different EPC populations. Whichever is the case, the results presented here show no association between the diverse EPC phenotypes described in the literature and the functional CFU-EPC assay.

5.3.3 Characterisation of endothelial progenitor cell colony forming (CFU-EPC) potential in subpopulations of mononuclear cells from haematopoietic stem cell sources and normal peripheral blood.

As an alternative to a phenotype definition, Hill *et al.*, 2003 have proposed that EPC may be quantified by their ability to form colonies under appropriate culture conditions (CFU-EPC). Here we have tried to identify the EPC present in adult tissues, by enriching for the different putative cell populations to establish which subpopulation is associated with CFU-EPC potential. Cells were selectively separated to high purity by plastic adherence or cell sorting using MACS or flow cytometry. Cells were phenotyped and CFU-EPC forming ability was tested in the Hill assay. We have shown that $CD34^+$ cells in the absence of other cell types were unable to proliferate or generate CFU-EPC in CECM media and that the cells died after a short time in culture. $CD133^+$-enriched cells behaved very differently than $CD34^+$-enriched cells even though $CD133^+$ cells partially over lap with $CD34^+$ cells. Despite not being able to generate CFU-EPC colonies, 90% of the surviving $CD133^+$ cultured cells presented a spindle-shaped morphology and stained positive for DiI-Ac-LDL. In contrast to our expectation, of all the cell fractions tested $CD14^+$-enriched plastic adherent cells gave rise to the highest number of CFU-EPC. These CFU-EPC outgrowths maintained CD14 and CD45 expression in culture and were also positive for expression of UEA-1 receptor, and VE-cadherin and took up DiI-Ac-LDL. However, they were negative for CD34 expression.
Initial discoveries of the existence of EPCs in the adult described the formation of adherent endothelial cell precursors from enriched $CD34^+$ cells (Asahara *et al.*, 1997). $CD34^+$-enriched MNCs were also injected in mice with unilateral hind limb ischaemia, and EPCs were localised after 1-6 weeks mainly in the capillaries of the neovascularisated ischaemic tissue (Asahara *et al.*, 1997). However, since the authors did not use pure $CD34^+$ cells (only 15% $CD34^+$ cells), the EPC precise origin in their

cultures remains unclear. In fact, the highest proportion of EPCs was obtained when the $CD34^+$-enriched population was cultured in the presence of CD34-negative cells, containing high percentage of peripheral blood $CD14^+$ cells. The relationship between EPCs and monocytes is complex. There clearly seem to be non-monocytic EPCs, but there also appear to be monocytes that have functional characteristics of CEC and EPCs. Harraz *et al.*, (2001) questioned whether $CD34^+$ cells (being only 0.1% of the total leucocytes) and as yet as many 10% of endothelial cells in the mouse neovasculature are blood derived cells, whether such small population could have such a profound effect on neovascularisation.

Since monocytes traverse the vascular wall during injury, share many antigenic characteristics with endothelial cells, and are responsive to vascular endothelial growth factor (VEGF), Harraz *et al.*, (2001) considered the possibility that monocytic cells might also function as EC progenitors. Monocytes could represent an intermediate phenotype in the endothelial pathway, which leads to mature endothelial cells. Camargo *et al.*, (2003) showed by lineage tracking, that myeloid cells are the haematopoietic stem cell–derived intermediates, which contribute to muscle regeneration.

CD14 is a well-known monocyte marker. Monocytes are thought to be mature end-stage cells with a limited potential to proliferate in culture. However, here some cells within the $CD14^+$ cells enriched by 2h plastic adherence, magnetic bead or flow cytometry sorting have been shown to have high proliferative potential. After only 3 days they formed relatively large CFU-EPC colonies.

$CD14^+$ cells were also shown to differentiate to dendritic cells when cultured in the presence of appropriate cytokines. These cells had typical dendritic cell morphology and phenotype. When the CFU-EPC potential of these dendritic cells was assessed in the Hill assay no colonies were detected.

Thus the $CD14^+$ cell population may be extremely flexible, adapting to changes in the external microenvironment. Ingram *et al.*, (2005) hypothesised that these cells could represent a unique form of macrophage differentiation, the angiogenic macrophage, indicating a possible common origin for monocyte-macrophage and endothelial lineages. Alternatively, it is possible that this is a heterogeneous population comprising several independent cell types including $CD14^+$ endothelial cell precursors with a unique proliferative capacity. To determine the adaptability or heterogeneity of this cell population will require further experimentation utilising $CD14^+$ cells derived from a single precursor cell.

Whichever is the case, only a specific subset of $CD14^+$ cells, whether true monocytes or independent endothelial precursors, act as stem cells with the ability to generate

endothelial cells (Zhao *et al.*, 2003). Although sorting of $CD14^+$ cells increased the frequency of CFU-EPC formation the proportion of the enriched cells able to generate CFU-EPC colonies was still low.

Subfractions of the $CD14^+$-enriched 2h plastic adherent cells; $CD14^+CD45^{dim}$, $CD14^+CD45^{brigh}$, $CD14^+$VE-cadherin$^+$, $CD14^+$Ve-cadherin$^-$, $CD14^+VEGFR2^+$ and $CD14^+VEGFR2^-$ were isolated by flow cytometry sorting and put through the CFU-EPC assay. $CD45^+$ is a haematopoietic marker that is thought to be lost with endothelial maturation. However, both $CD14^+$CD45 bright and dim subfractions formed CFU-EPC without major differences. Similar results were seen for the other separations. Elsheikh *et al.*, (2005) reported that peripheral blood monocytes co-expressing $CD14^+VEGFR2^+$ but not $CD14^+VEGFR2^-$ monocytes had the potential to differentiate *in vitro* into cells with endothelial characteristics and contributed significantly after injury to the re-endothelialisation of femoral arteries. In our hands, sorted $CD14^+VEGFR2^+$ and $CD14^+VEGFR2^-$ both generated comparable numbers of CFU-EPC.

Recently, Romagnani *et al.*, (2005) reported a peripheral blood-derived cell population with both endothelial and stem cell properties including high expression of mRNA for Nanog and Oct4 suggesting multipotent properties. These $CD14^+$ cells were negative for CD34 expression by conventional cytoflurometric techniques but were found to express low levels of CD34 when assessed by the highly sensitive antibody-conjugated magnetofluorescence amplification (ACMFL).

In our study attempts to isolate $CD14^+CD34^-$ and $CD14^+CD34^{low}$ cell fractions generated too few cells to assess their potential in the Hill assay.

Clanchy *et al.*, (2006) showed recently that a subpopulation(s) of human monocytes could proliferate *in vitro* in response to M-CSF. Presumably, these proliferative monocytes are less mature than the other monocytes. Carboxyfluorescein diacetate succinimidyl ester (CFSE) labelling enabled the estimation of the number of peripheral monocyte divisions over time. Following CFSE labelling and culture, proliferative monocytes have shown to have a distinctive, spindle-shaped morphology compared to non-proliferating population (Clanchy *et al.*, 2006). However, these authors have not linked any monocyte subpopulation with endothelial properties. Future experiments will attempt to sort proliferative and non-proliferative $CD14^+$ cells and assess their endothelial colony forming capacity and phenotype.

5.3.3.1 Putative EPC sources

Bone marrow, cord blood and G-CSF mobilised peripheral blood are sources rich in CD34 and CD133 positive HSC which have been proposed as a possible common precursor of EPC. In this study, results from the Hill assay showed that of these, mobilised peripheral blood gave almost no colonies and cord blood gave few colonies. Bone marrow had the highest frequency of CFU-EPC, but this was sill lower than that achieved using HSC poor normal peripheral blood. Further, bone marrow and cord blood colonies were much smaller and morphologically different from those produced from peripheral blood. Whether it is because bone marrow and cord blood contain less mature circulating CFU-EPC compared to normal peripheral blood is unknown. These results, with those of experiments using $CD34^+$ or $CD133^+$ cell populations suggest that HSC are not the cell population directly responsible for CFU-EPC.

The data presented here is the first to show that immediately following G-CSF treatment peripheral blood from healthy donors and patients is virtually unable to generate CFU-EPC. Further investigations showed that prior to G-CSF administration and 2 months following completion of treatment samples from healthy donors showed good CFU-EPC potential, which was virtually abolished immediately following G-CSF administration. This would be a cause of concern regarding the use of G-CSF mPB for patients requiring endothelial reconstitution. Moreover, a dramatic decrease in CFU-EPC numbers was seen after the exogenous addition of G-CSF (see 5.2.3.10).

Unlike our results, Powell *et al.*, (2004) showed a 10-fold EPC colony-forming units (CFU) increase over baseline measurements after G-CSF administration in patients with coronary artery disease. However, similar to our results they showed an increase co-expression of $CD34^+/CD133^+$ haematopoietic surface markers in samples after G-CSF administration (see 5.2.2.4 and 5.2.3.10.2). Powell *et al.*, (2004) also reported that there was no correlation between numbers of CD133/VEGFR2 cells measured by flow cytometry and EPC colony-forming units in the culture, despite the increase in circulating cells after G-CSF. Differences in CFU-EPC potential between our data and Powell *et al.* data may be due to the two different patient groups analysed.

We are confident in our results obtained in the G-CSF mobilised samples as in all the cases a dramatic decrease in CFU-EPC was found compared to non-mobilised samples. Powell *et al.* started from a population of coronary artery patients where they originally have a reduced CFU-EPC potential compared to healthy individuals. Therefore, the CFU-EPC capacity starting levels were different in the two studies.

G-CSF mobilised samples showed similar flow cytometry numbers of $CD14^+$ cells

by comparison to peripheral blood, cord blood and bone marrow with no significant differences between all the $CD14^+$ subpopulations studied. 30 CFU-EPC/10^6 cells plated were counted in samples before G-CSF administration. Thus, it means that only 1 in 33,000 cells had the potential to proliferate and generate endothelial colonies. In most cases 50,000 events were collected in each sample by flow cytometry and even if 100,000 had been collected this would reflect less than 4 cells in total. Therefore, any change of expression of these 4 cells in samples prior or after G-CSF administration would have been below our limits of detection. How G-CSF administration negatively affects CFU-EPC generation is currently being studied. Also intriguing is the diminished colony potential in the Hill assay seen in cord blood samples by comparison with bone marrow and even more strikingly to normal peripheral blood samples. Ingram *et al.*, (2005) suggested that cord blood and peripheral blood EPCs could be derived from different populations of progenitors. Lin *et al.*, (2000) demonstrated outgrowth from CD14-negative cells of typically endothelial cells (OEC) with long-term proliferative capacity. An increase of 15-fold in this "late" colony potential was found in cord blood samples when compared to peripheral blood colonies. CFU-EPC potential measured by Hill's assay may detect early EPCs, and although cord blood may contain late outgrowth endothelial cells it may not contain early EPCs which are being detected in peripheral blood and bone marrow.

Currently EPC are defined as a bone marrow derived cells which incorporate into sites of ischaemia and neovascularise damaged tissue. This is a general description based on the outcomes of clinical trials where unselected, total bone marrow or bone marrow mononuclear cells have been used rather than specific cell fractions. It includes cells expressing CD34 and/or CD133, $CD14^+$ adherent cells or mesenchymal stem cells (MSC), therefore it is impossible to say which cell is responsible for any beneficial effect seen.

The culture of unprocessed MNCs is one of the most common used methods to obtain EPC (Tepper *et al.*, 2002) and since adherence to the culture plate is an intrinsic property of EPCs, this method could be used to obtain EPCs regardless of their real origin (Zhang *et al.*, 2006). Cytofluorimetric techniques have shown that these adherent cells, considered as EPCs by a number of groups, consist of a cell population which shares monocytic markers and EC markers (Romagnani *et al.*, 2005; Kalka *et al.* a), 2000; Rehman *et al.*, 2005; Zhang *et al.*, 2006). All these cells were positive for endothelial markers such as ac-LDL, Ulex lectin (90%), CD31, and CD105; but also positive for haematopoietic and monocyte/macrophage markers: CD45, CD14, CD11c, CD16, CD86 and negative for CD34 or CD133 markers (Romagnani *et al.*,

2005). Similarly, Kalka *et al.* a), (2000) showed that 90% of these primary adherent EPCs after 7 days of *ex vivo* expansion also expressed CD14 monocyte marker. Rehman *et al.*, (2003) showed that MNCs after 4 days culture plated in fibronectin coated flasks, were positive for ac-LDL and Ulex lectin, but also positive for CD45, CD14 (95%) and CD11c (90.8%). Therefore, experiments that have defined EPCs only as cells positive for ac-LDL and Ulex-lectin in fact fail to add that they were also probably positive for the expression of monocyte/macrophage markers. Further Zhang *et al.*, (2006) believes that the so-called EPCs obtained by *in vitro* culture of MNCs are in fact monocytes derived from $CD34^+$ haematopoietic cell, which can express endothelial characteristics. These monocytes as well as being positive for ac-LDL uptake performed a phagocytosis function of leucocytes/monocytes maintaining their macrophage function throughout the *in vitro* culture (Zhang *et al.*, 2006). Conversely, Peichev *et al.*, 2000 demonstrated that $CD34^+VEGFR2^+CD133^+$ primary non-adherent endothelial precursors mature to endothelial cells and did not show any expression of CD14. Thus, a second population of CD14-negative cells seems to exist which is capable of forming endothelial colonies of long-term proliferative capacity, referred as late outgrowth endothelial cells (OECs). Due to their long term proliferation, these cells could provide higher cell counts typically desired in tissue engineering applications (Zisch, 2004).

In summary, as measured by the Hill assay for CFU-EPC, the cell population able to proliferate and generate colonies at the highest frequency are adherent $CD14^+$ cells. Though immediately following *in vivo* G-CSF treatment this ability was lost.

5.3.3.2 *Ex vivo* expansion

Previous studies on myeloid precursors (Chapter 3) showed that $CD34^+$ cells expand up to 15-20-fold after 14 days culture in a simple SCF/Flt-3L cytokine combination. We have studied the further addition of known endothelial growth factors as VEGF, bFGF and others to evaluate cell expansion and endothelial differentiation. Repeated experiments showed that none of these factors had any adverse effect on the cell expansion numbers, but no significant benefit either.

Using expression of VEGFR2 as an index of endothelial differentiation it was found that the addition of VEGF together with SCF and Flt-3L increased the number of $VEGFR2^+$ cells after 14 days as compared to that when VEGF was absent. Also the addition of heparin in the SCF/Flt-3L expansion cocktail promoted endothelial differentiation with an increase of the number of $VEGFR2^+$ cells/ml after culture, though this did not achieve significance.

Commercially available media specifically formulated for culture of mature

endothelial cells and the use of fibronectin or gelatin coated plates were ineffective in increasing the number of VEGFR2$^+$ cells derived from CD34$^+$ enriched cells as compared to controls cultured in basic cell media.

After 14 days culture with the SCF/Flt-3L+VEGF cytokine cocktail, about 24% of the cells ($0.3x10^6$ cells/ml) expressed VEGFR2. Similar numbers were achieved in terms of VE-cadherin and UEA-1 expression. Moreover, cultured cells generated outgrowth endothelial colonies and cord structures. These cells were also positive for DiI-Ac-LDL staining.

To investigate the relationship if any, between CD34$^+$ and CD133$^+$ cells and CD14$^+$ cells, the generation of CFU-EPC by CD14$^+$ cells derived *ex vivo* from CD34$^+$ cells from all the sources was tested. CD34$^+$ cells were enriched and cultured with cytokines to generate CD14$^+$ cells in first instance, which were then used in the Hill assay for comparison with the results from CD14$^+$ cells isolated directly from all sources tested. The generation of CD14$^+$ cells from CD34$^+$ cells was achieved from all sources and the cells were characterised by flow cytometry. In the Hill assay, spindle-shaped cells and some CFU-EPC colonies were seen using cells derived from all sources, though the colonies were smaller by comparison to those detected when CD14$^+$ adherent cells were used. Importantly, the CD14$^+$ cells derived by *ex vivo* expansion of CD34$^+$ cells from G-CSF mobilised peripheral blood and cord blood generated spindle-shaped cells and CFU-EPC after 2 weeks of culture. CD14$^+$ cells could represent an intermediate phenotype in the endothelial pathway, which leads to mature endothelial cells. Therefore CD34 and/or CD133$^+$ cells could be the original cell population which after culture develops into an intermediate CD14$^+$ cell able to generate CFU-EPC. Whether the CD14$^+$ cell has a limited proliferative potential compared to a more multipotent CD34$^+$ and/or CD133$^+$ is still not resolved.

It is not known if there is any relationship between mesenchymal stem cells (MSC) and CD14$^+$ cells. Putative MSC isolated from peripheral blood were able to generate CFU-EPC after 5-day culture in endothelial culture media comparable to that achieved using selected CD14$^+$ cell populations. Further, following the primary fibronectin adherence step in the Hill assay, cells remaining non-adherent, cultured in conditions optimal for expansion of MSC could be differentiated along the osteogenic lineage.

Thus, whether a single cell has the potential to act as a mesenchymal and an endothelial progenitor cell exist in the progenitor cell or same starting material remains an aim of further study.

Chapter 6

Endothelial progenitor cell frequencies in peripheral blood: clinical studies

6.1 Introduction

The traditional paradigm of vascular repair is based on the proliferation and migration of pre-existing mature endothelial cells from the adjacent vasculature (Risau, 1995). The discovery by Asahara and colleagues that mononuclear cells in peripheral blood have the potential to differentiate into endothelial cells has launched a new field of cardiovascular research (Asahara *et al.*, 1997). As discussed in Chapter 5, endothelial progenitor cells (EPCs) are increasingly characterised by their expression of both haematopoietic (CD34 and/or CD133) and endothelial cell markers VEGFR2 (also known as KDR), but a definitive phenotype remains elusive. These putative EPCs or their progeny form vascular structures *in vitro* and are incorporated into the vessel wall in experimental models of neovascularisation (Murohara *et al.*, 2000). These cells may have an important role in the maintenance and repair of the vascular endothelium, and in the pathogenesis of atherosclerotic plaque formation and its consequences. A number of recent experimental and clinical studies have revealed that ischaemic heart diseases and peripheral vascular disease can effectively be treated by autologous EPC transplantation into affected tissue as presented in 5.1.17 section of the Introduction.

Endothelial progenitor cells can be isolated and cultured from a variety of cell populations in peripheral blood and bone marrow, but as yet no definitive phenotype has been ascribed to EPCs. Comparisons between clinical studies have been limited by the use of a variety of phenotypic markers to discriminate EPCs and by the lack of comparable functional assays. In the face of an uncertain phenotype, the EPC colony forming unit assay (CFU-EPC) has emerged as an alternative specific enumeration system for EPCs (Hill *et al.*, 2003). Although groups are increasingly

quantifying either phenotypic EPCs ($CD34^+KDR^+$) or functional CFU-EPCs, few clinical studies had reported both or commented on the relationship between phenotype and function at the time when we undertook these studies.

Using various parameters of measurement, endothelial progenitor cells have been reported to be infrequent in peripheral blood. In cardiovascular diseases they may be even lower, but numbers seem to increase in response to myocardial ischaemia and acute myocardial infarction (Adams *et al.*, 2004; Massa *et al.*, 2005). Reduced numbers of EPCs have been demonstrated in cigarette smokers (Vasa *et al.*, 2001), patients with diabetes mellitus (Fadini *et al.*, 2005), and in those with evidence of endothelial dysfunction (Herbrig *et al.*, 2006). These patients are at high risk of complications following percutaneous coronary intervention (PCI). Furthermore, patients with diffuse in-stent re-stenosis have reduced EPC number and function in comparison with matched controls at the time of presentation (George *et al.*, 2003). Inadequate EPC number and function prior to angioplasty, as well as inadequate early and sustained EPC recruitment, may favour a maladaptive response to arterial injury and result in an increased incidence of in-stent thrombosis, re-stenosis and ischaemic complications. The immediate effects of local vascular injury during angioplasty and stenting on the mobilisation of EPCs are not known.

Patients with type 1 diabetes experience poor wound healing secondary to a reduced capacity to form collateral vessels in areas of tissue ischaemia (Tamarat *et al.*, 2004). Until recently the reasons for this have remained unknown. However, with the identification of bone marrow-derived endothelial progenitor cells (EPCs), it is now thought that the quantitative and qualitative changes seen in this cell group in patients with both type 1 and type 2 diabetes are critical to the process of impaired neovascularisation (Loomans *et al.*, 2004; Asahara *et al.*, 1997; Fadini *et al.*, 2005; Awad *et al.*, 2005).

Ischaemic heart disease is a major cause of morbidity and mortality worldwide. Despite advances in percutaneous coronary intervention (PCI), major adverse cardiac events occur in up to 30% of patients following balloon angioplasty and 20% following stenting (Serruys *et al.*, 1994). Vascular trauma, induced by percutaneous intervention, initiates a sequence of events in which the release of cytokines and growth factors result in the proliferation of smooth muscle and deposition of platelets and leucocytes at the site of injury, accelerating vascular repair. Endothelialisation is necessary to prevent mural thrombus formation and neointimal hyperplasia that may otherwise lead to ischaemic complications and restenosis.

The aim of these clinical studies presented in this last chapter was to measure both circulating phenotypic EPCs by flow cytometry analysis, functional CFU-EPCs by

cell culture and endothelial cell marker message mRNA by real-time PCR following a) PCI in patients with stable coronary disease b) elective abdominal aortic aneurysm repair in patients as a model of tissue ischaemia and blood vessel damage and c) in type 1 diabetes patients.

6.2 Results

Clinical assessments, interventions and blood sampling, were carried out by Dr. Nick Mills (Centre for Cardiovascular Science, Edinburgh University).
Total and differential blood cell counts and serum C-reactive protein concentrations were measured in the Department of Haematology, University of Edinburgh).
Leucocyte m-RNA values for specific genes were analysed by Dr. Chris Millar, Department of Haematology, University of Edinburgh).
Peripheral blood phenotyping by flow cytometry and endothelial colony assays (CFU-EPC) on all samples were carried out by me.

6.2.1 Coronary angiography and percutaneous coronary intervention (PCI) (Study in collaboration with Dr. Nick Mills(Centre for Cardiovascular Science,Edinburgh University)

There were no complications arising from angiography or PCI and all patients (Table 6.1) were discharged home 24 hours after procedure.

6.2.1.1 Inflammation and myocyte necrosis

Diagnostic angiography did not increase peripheral blood leucocyte count or serum C-reactive protein concentrations (Table 6.2). Coronary intervention increased neutrophil count (D1.31±0.35x10^9/L, P<0.001) and serum C-reactive protein concentrations (D2.5±1.5mg/L, P=0.001) at 24-hours. There was an apparent transient significant reduction in monocyte count immediately following catheterisation alone (D-0.08 ±0.05x10^9/L, P=0.15) that was present after PCI (D-0.10±0.0.3x10^9/L, P=0.005). Monocyte count returned to pre-procedure levels by 24 hours. There was no evidence of significant myocyte necrosis 24 hours following diagnostic angiography or PCI.

6.2.1.2 Endothelial progenitor cells

Mononuclear cells plated on fibronectin formed typical colony forming units (CFU-EPCs), characterized previously as a central cluster of rounded cells surrounded by radiating thin, flat cells (Hill *et al.*, 2003) . Although unaffected by diagnostic

angiography, the number and cellularity of CFU-EPCs were increased 24 hours after PCI (Figures 6.1 and 6.2). Direct staining confirmed that CFU-EPCs, like mature endothelial cells, bound *ulex europaeus* lectin and integrated acetylated-LDL. The majority of both small round cells and spindle shaped cells expressed CD14 (See 5.2.3.5)

The increase in CFU-EPCs or functional endothelial progenitor cells was not associated with an increase in the number of circulating $CD34^+KDR^+$ cells (Table 6.3). Interestingly there was no correlation between EPCs identified by phenotype ($CD34^+KDR^+$ cells) and the number of functional EPCs quantified using cell culture (CFU-EPCs) either before ($r=-0.15$, $P=0.58$) or 24 hours ($r=0.02$, $P=0.94$) after angiography. Similarly, there was not a significant increase in leucocyte CD34 mRNA following angiography or PCI (Table 6.4). There was however, a significant reduction in the leucocyte CD14, CD34, VE-cadherin, and vWF mRNA immediately after catheterisation in both diagnostic and interventional studies, which coincided with the reduction in circulating monocytes. Relative quantities of leucocyte mRNA were restored 24 hours following catheterisation, with only CD14 mRNA increasing following PCI ($P<0.05$).

	Angiography	PCI
	n=20	n=20
Age (years)	62±2.	59±3
Sex (male/female)	11/9	14/6
Cigarette smokers	4	3
Diabetes mellitus	7	2
Hypertension	12	12
Previous MI	2	3
Previous PCI	1	7
Prior CABG	0	1
Total cholesterol(mg/dL)	193±15	172±7
LDL-cholesterol(mg/dL)	102±15	92±8
HDL-cholesterol(mg/dL)	45±3	46±3
Triglycerides(mg/dL)	224±26	189±34
Fasting Glucose (mg/dL)	151±18	100±4
C-reactive protein(mg/L)	3.9±0.7	2.5±0.8
Number of diseased vessels		
Normal/minor disease	12	0
1 vessel disease	0	9
2 vessel disease	4	8
3 vessel disease	4	3
Stent implantation		
De novo lesion	0	17
Restenosis	0	3
Drugs		
Aspirin	13	16
Clopidogrel	20	20
Statin	10	18
ß-blocker	12	17
ACE inhibitor/ARB	10	12

Values are presented as number or mean ± SEM

Table 6.1

Clinical characterisation and angiographic findings of patients undergoing diagnostic angiography or percutaneous coronary intervention

	Pre-procedure	Post-procedure	6 hours	24 hours
Angiography				
Leucocytes ($x10^9$/L)	6.5 ± 0.3	6.7 ± 0.4	6.9 ± 0.4	6.3 ± 0.4
Neutrophils ($x10^9$/L)	3.8 ± 0.2	4.3 ± 0.4	4.2 ± 0.3	3.7 ± 0.3
Lymphocytes ($x10^9$/L)	1.9 ± 0.1	1.7 ± 0.1	1.9 ± 0.2	1.8 ± 0.2
Monocytes ($x10^9$/L)	0.58 ± 0.05	0.51 ± 0.04	0.61 ± 0.09	0.59 ± 0.09
C-reactive protein (mg/L)	3.9±0.7	-	-	4.6±0.7
PCI				
Leucocytes ($x10^9$/L)	7.1 ± 0.4	6.7 ± 0.3	7.2 ± 0.2	8.2 ± 0.3*
Neutrophils ($x10^9$/L)	4.4 ± 0.3	4.2 ± 0.3	4.5 ± 0.2	5.7 ± 0.3†
Lymphocy tes ($x10^9$/L)	1.8 ± 0.1	1.7 ± 0.1	1.9 ± 0.1	1.7 ± 0.1
Monocytes ($x10^9$/L)	0.58 ± 0.03	0.48 ± 0.04*	0.58 ± 0.02 ‡	0.56 ± 0.03‡
C-reactive protein (mg/L)	2.5±0.8	-	-	5±1.6*

Values ar e presented as mean ± SEM
*P<0.05, †<0.001 pre-procedure versus time point
‡P<0.05 post-procedure versus time point

Table 6.2 Markers of inflammation following diagnostic angiography or percutaneous coronary intervention

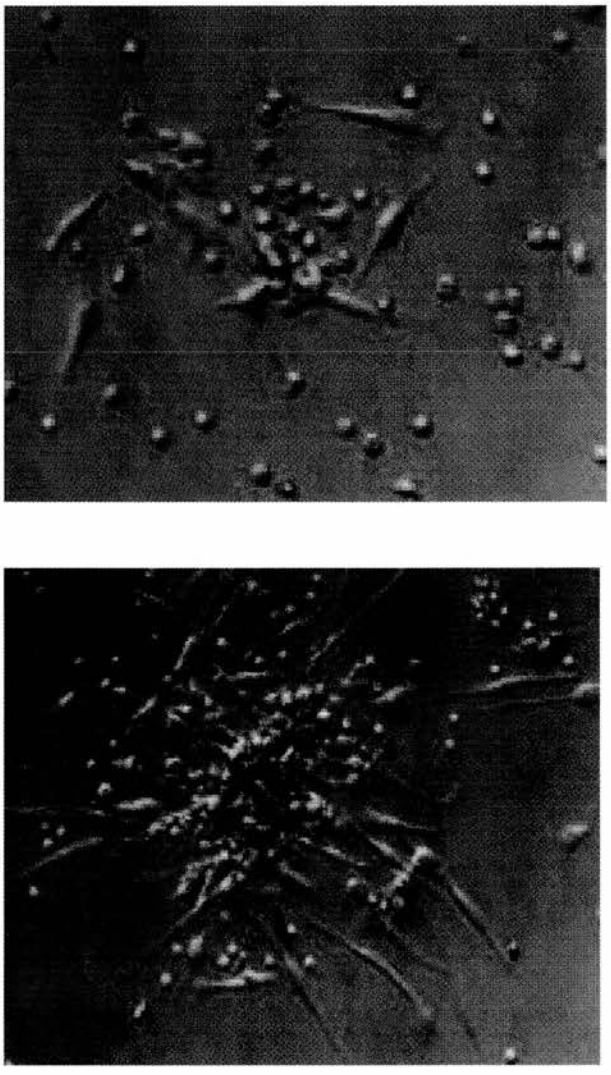


Figure 6.1 Phase contrast images of CFU-EPC detected before and after PCI

Phase contrast microscopy of a typical CFU-EPC (a) before and (b) after 24h after coronary angioplasty and stenting.

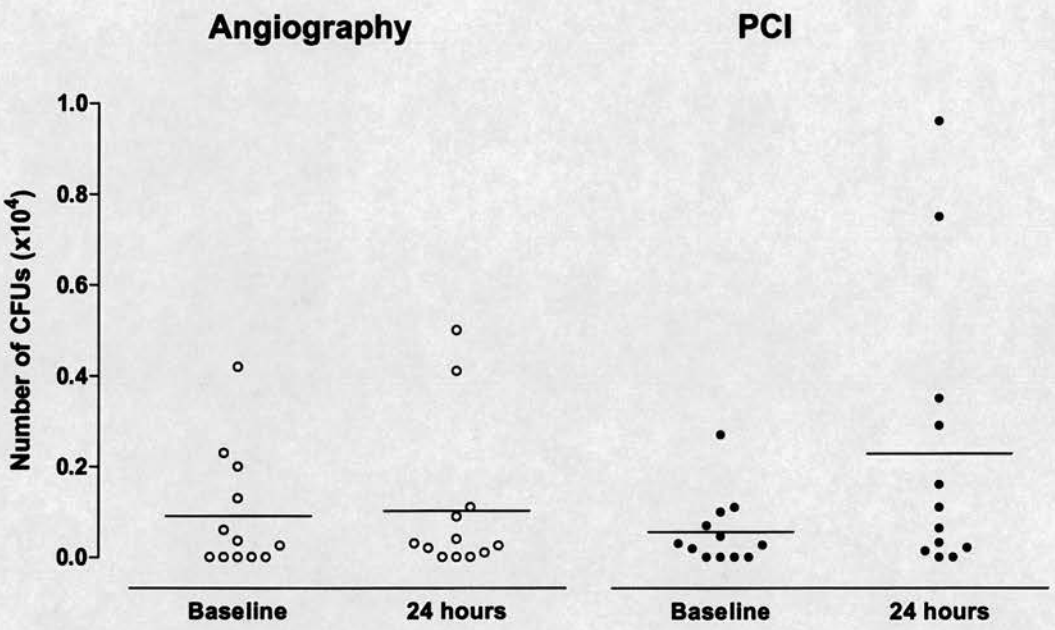


Figure 6.2 Frequency of CFU-EPC detected before and after diagnostic angiography or PCI

Number of CFU-EPCs before and 24h following diagnostic angiography (P=0.8) or PCI (P=0.05).

	Pre-procedure	Post-procedure	6 hours	24 hours
Angiography				
CD34+ cells (% leucocytes)	0.100 ± 0.019	0.082 ± 0.015	0.087 ± 0.016	0.102 ± 0.028
CD34+KDR+ cells (% leucocytes)	0.030 ± 0.010	0.020 ± 0.005	0.021 ± 0.004	0.026 ± 0.007
CD34+ cells (x 10^3/mL blood)	6.54 ± 0.92	5.43 ± 0.80	5.98 ± 0.81	5.67 ± 1.15
CD34+KDR+ cells (x 10^3/mL blood)	2.11 ± 0.69	1.45 ± 0.41	1.47 ± 0.26	1.52 ± 0.38
PCI				
CD34+ cells (% leucocytes)	0.083 ± 0.011	0.105 ± 0.011	0.091 ± 0.012	0.083 ± 0.010
CD34+KDR+ cells (% leucocytes)	0.019 ± 0.003	0.029 ± 0.006	0.020 ± 0.004	0.021 ± 0.004
CD34+ cells (x 10^3/mL blood)	5.40 ± 0.61	7.00 ± 0.82	6.33 ± 0.84	6.55 ± 0.76
CD34+KDR+ cells (x 10^3/mL blood)	1.15 ± 0.19	1.91 ± 0.47	1.28 ± 0.29	1.65 ± 0.36

Values are presented as mean ± SEM

Table 6.3 Circulating $CD34^+KDR^+$ cells following angiography or percutaneous coronary intervention

	Pre-procedure	Post-procedure	6 hours	24 hours
Angiography				
CD34	1.7 ± 0.3	1.1 ± 0.2*	1.6 ± 0.3	2.5 ± 0.8
CD14	3.1 ± 0.4	2.1 ± 0.3*	2.2 ± 0.3	2.9 ± 0.5
vWF	2.5 ± 0.5	1.7 ± 0.2*	1.8 ± 0.4	2.3 ± 0.7
VE-cadherin	4.4 ± 2.0	2.8 ± 1.3*	3.3 ± 1.7	6.9 ± 4.6
PCI				
CD34	2.3 ± 0.5	1.6 ± 0.5*	1.9 ± 0.4	2.1 ± 0.4
CD14	3.3 ± 0.5	2.1 ± 0.4*	3.1 ± 0.7	4.7 ± 1.0‡
vWF	2.3 ± 0.4	2.0 ± 0.4*	1.9 ± 0.3	2.5 ± 0.5
VE-cadherin	1.5 ± 0.3	0.9 ± 0.2 †	1.3 ± 0.3	1.5 ± 0.4

Values are presented as relative quantities, mean ± SEM
*P<0.05, †<0.001 pre-procedure versus time point
‡P<0.05 post-procedure versus time point

Table 6.4 Leukocyte mRNA levels following angiography or percutaneous coronary intervention

6.2.2 Aortic Aneurysm repair

(Study in collaboration with Dr. Chris Millar, Department of Haematology, University of Edinburgh).

6.2.2.1 Comparison between patients prior to aortic aneurysm repair and control subjects

Prior to surgery differences between the patient group and control subjects were analysed. The mean number of functional CFU-EPCs was significally lower in patients by comparison to that detected in the control group (Figure 6.3). In contrast flow cytometry measurements of CD34, CD133, VEGFR-2 and VE-cadherin did not differ significally in patients prior to aortic aneurysm compared to the control group (table 6.5). Furthermore, there was no significant difference between the patient group and control group in the relative quantity of mRNA for CD34, VEGFR-2, VE-cadherin and vWF (table 6.6).

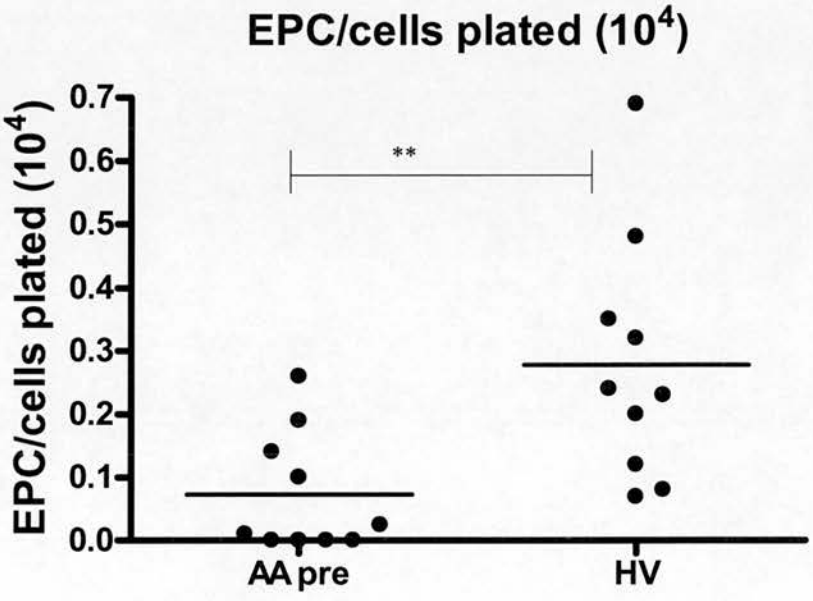


Figure 6.3. Comparison of median colony numbers and median percentage positivity of EPCs in control versus pre-treatment patient group.

The control group was not cohort matched with the patient group. The control group had a higher median number of cell colonies ($0.27x10^4 \pm 0.06x10^4$) compared with the patient group ($0.07x10^4 \pm 0.03x10^4$). This difference was statistically significant ($p=0.005$).

	Control gp (n=15)	Patients (n=15)
Age		
Mean	50.5	70.3
p value	0.003	
EPC/cells plated (10^4)		
Median	0.27	0.07
p value	0.0052	
% CD34+ cells		
Median	0.065	0.06
p value	0.7	
% CD133+ cells		
Median	0.07	0.08
p value	0.93	
% CD34+VEGFR2+ cells		
Median	31.1	26.2
p value	0.62	
% VEGFR2+ cells		
Median	1.66	4.24
p value	0.18	
% VE-cadherin+ cells		
Median	8.415	13.07
p value	0.08	

Table 6.5. There were no statistically significant differences between the groups in the median percentage of cells expressing CD34 (p=0.7), CD133 (p=0.93), VEGFR2 (p=0.18) and VE-cadherin (p=0.08)

Target	Control group A	Patient group
	n=12	n=15
Age		
Mean	71	70.3
p value	0.82	
CD34		
Median	2.538	0.984
p value	0.237	
VEGFR2		
Median	2.089	1.599
p value	0.6454	
VE-cadherin		
Median	3.373	2.498
p value	0.863	
vWF		
Median	2.363	2.435
p value	0.99	

Table 6.6. Comparison of median relative mRNA levels between the control group and the pre-treatment patient group.

The control group was cohort matched for age and gender with the patient group. There were no statistically significant differences found between the two groups in the RQ values for CD34 mRNA (p=0.237), VEGFR2 (p=0.6454), VE-cadherin (p=0.863) and vWF (p=0.999).

6.2.2.2 Leucocyte and CFU-EPC quantitation following surgery for aortic aneurysm repair.

6.2.2.2.1 Total white cell count and monocyte count. CD14 expression by PCR.

Patients demonstrated an increase in the mean total white cell count by 24 and 48 hours compared to pre-operative levels. This was followed by a return to pre-operative levels by day 5 (Figure 6.4a). There was also an increase, although no significant, in the mean monocyte count by 48 hours post-operatively(Figure 6.4b). Samples from 14 patients out of 15 patients were assessed for the relative quantity (RQ) of CD14 mRNA. There was an increase in CD14 mRNA levels by 24 and 48 hours, followed by a reduction by day 5 post-operatively (Figure 6.4c).

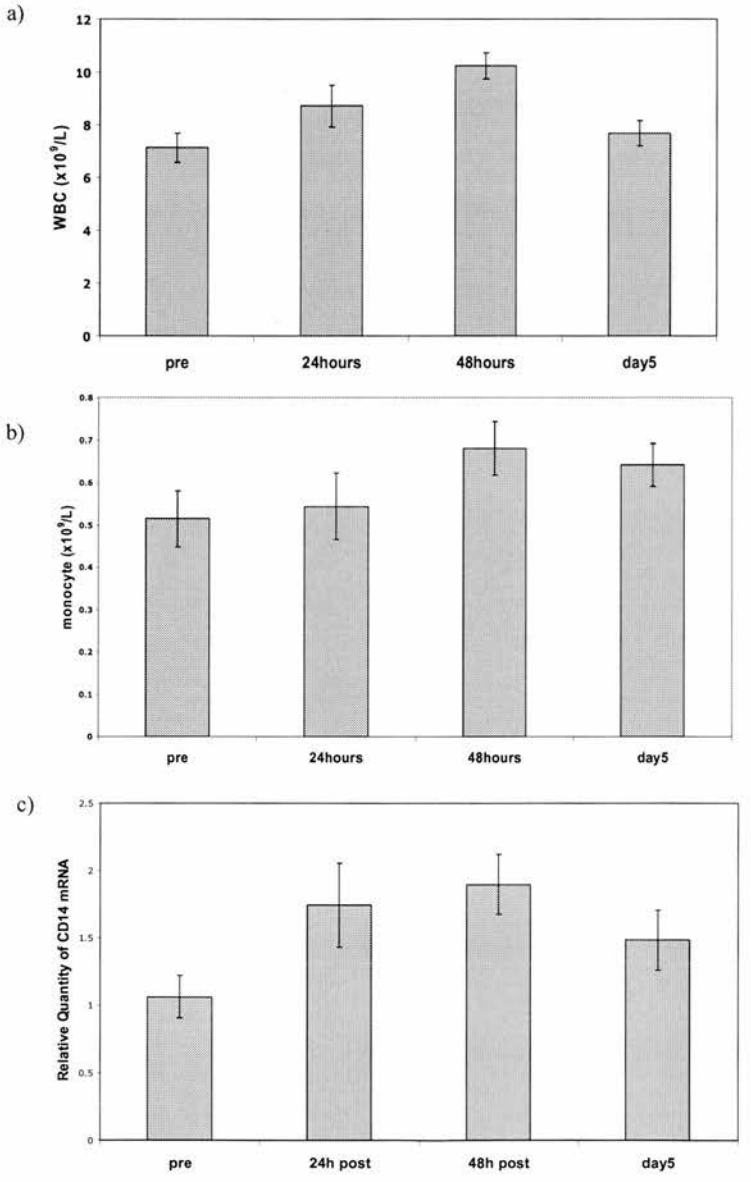


Figure 6.4. Total white cell count, monocyte count and CD14 expression by PCR.
Effect of aortic aneurysm repair on the total white cell count (WBC), the monocyte count and the RQ of CD14 mRNA. **(a)**. Mean (±SEM) WBC increases from a baseline of $7.13x10^9$/l (±0.55) to $8.71x10^9$/l (±0.79) by 24 hours, to a peak of $10.23x10^9$/l (±0.49) by 48 hours. The WBC then decreases by day 5 to $7.67x10^9$/l (±0.5) (p=0.0001). **(b)**. Mean (±SEM) monocyte count increases from a baseline of $0.51x10^9$/l (±0.07) to $0.68x10^9$/l (±0.06) by 48 hours. The monocyte count is $0.64x10^9$/l (±0.05) by day 5. The change in monocyte count is not significant (p=0.1). **(c)**. The mean (±SEM) RQ of CD14 mRNA mirrors the change in the mean WBC count. There is an increase from a pre-operative value of 1.06 (±0.16) to 1.74

6.2.2.2.2 CFU-EPC colony assay

Samples from 10 patients were assessed in the CFU-EPC colony assay. There was a transient reduction, although not significant, in the mean number of CFU-EPC colonies by 24 hours after the operation followed by an increase of CFU-EPC at 48h after procedure (Figure 6.5).

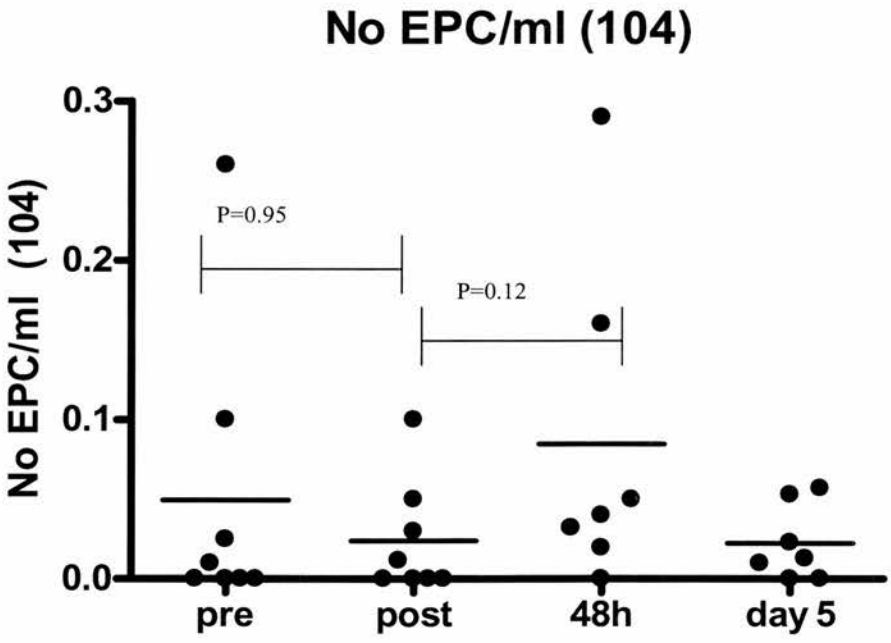


Figure 6.5 CFU-EPC colony assay
The median number of CFU-EPC in culture is reduced by 24 hours after the operation, but this does not reach statistical significance (p=0.41).

6.2.2.2.3 CD34 expression

There was a reduction, although not significant, in the median number of $CD34^+$ cells by 24 hours post-operatively, before a recovery to the pre-operative level by 48 hours (Figure 6.6a). In addition, there was a significant reduction in the median mRNA levels of CD34 by 24 hours post-operatively (p=0.03), with a recovery to the median pre-operative level by day 5 (p=0.03) (Figure 6.6b).

6.2.2.2.4 CD133 expression

Similarly, there was a significant reduction in the median number of $CD133^+$ cells by 24 hours (p=0.01), before a return to baseline levels by day 5 post operatively (p=0.007 day 5 versus 24h)(Figure 6.6c). There also was a significative increase in the CD133 mRNA by day 5 following the operation compared to that obtain at 24h (p=0.03)(Figure 6.6d).

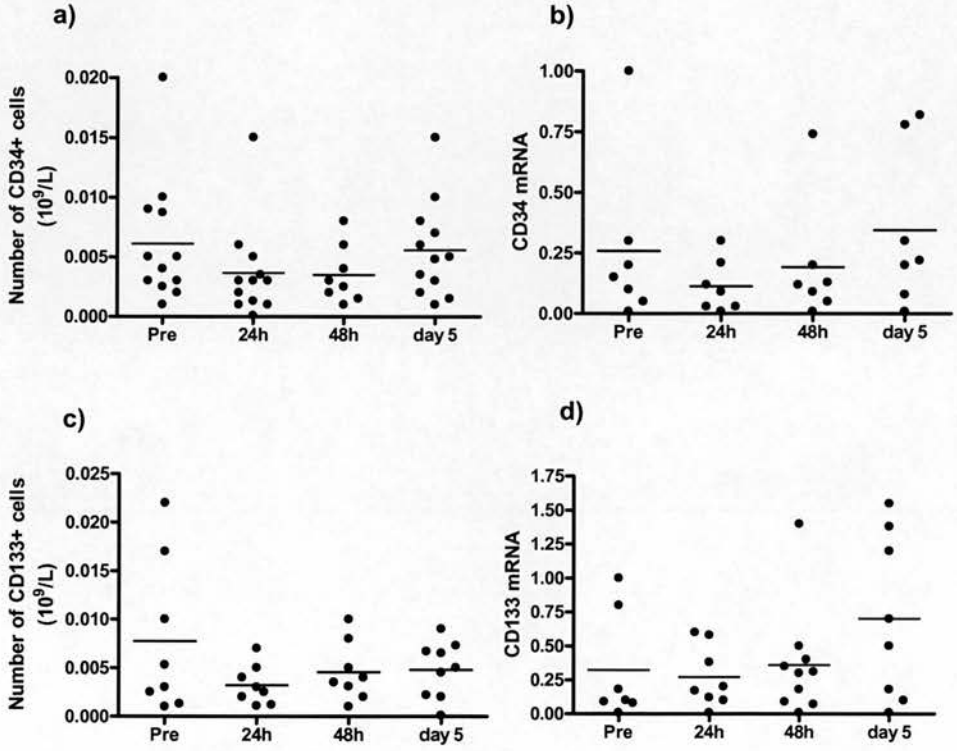


Figure 6.6 Effect of aortic aneurysm repair on markers of EPCs

a) There is a reduction in the median number of $CD34^+$ cells by 24 hours, with recovery by 48 hours, **b)** The median RQ of CD34 mRNA transiently decreases by 24 hours before recovering to baseline by 48 hours, **c)** the median number of $CD133^+$ cells transiently decreases by 24 hours after procedure and it returns to baseline or higher levels by day 5 after the operation, **d)** the median RQ of CD133 increases at 24h after operation and returns to baseline levels by day 5.

6.2.2.2.5 VEGFR-2 expression

Samples from 9 patients were assessed for the expression of VEGFR2. There was not a significance change in VEGFR2 protein surface expression or mRNA levels over procedure(data not shown).

6.2.2.2.6 Dual CD34 and VEGFR-2 expression

There was a transient reduction in the number of $VEGFR2^{+}$ CD34+ co-expressing cells by 24 hours after the operation (p=0.007) returning to baseline levels at day 5 post procedure (p=0.01 day 5 versus 24h)(Figure 6.7).

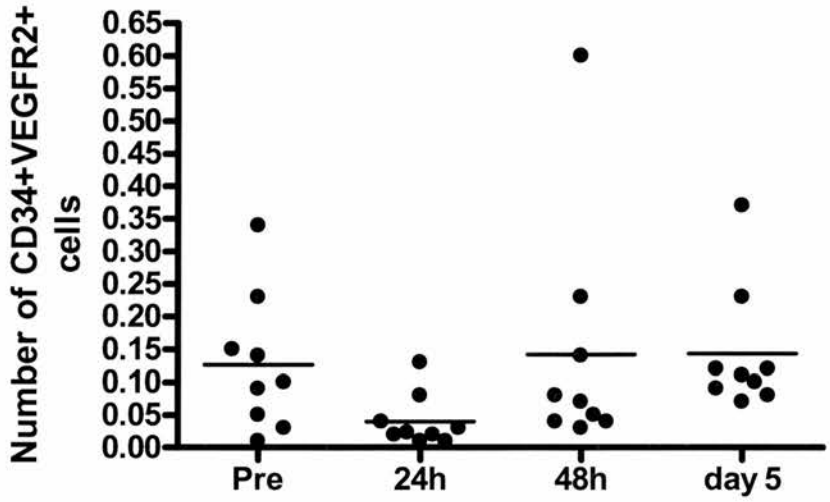


Figure 6.7 Effect of aortic aneurysm repair on EPCs, using dual staining of markers by flow cytometry.

When gated on all cells, the number of $CD34^{+}VEGFR\text{-}2^{+}$ co-expressing cells falls significantly by 24 hours post operatively, before recovering to baseline by day 5.

6.2.3 Diabetes mellitus

Study in collaboration with Dr. Chris Millar, Department of Haematology, University of Edinburgh).

The mean number of functional CFU-EPC was significally lower in patients with type I diabetes by comparison to that detected in the control group (p=0.004) (Figure 6.8).

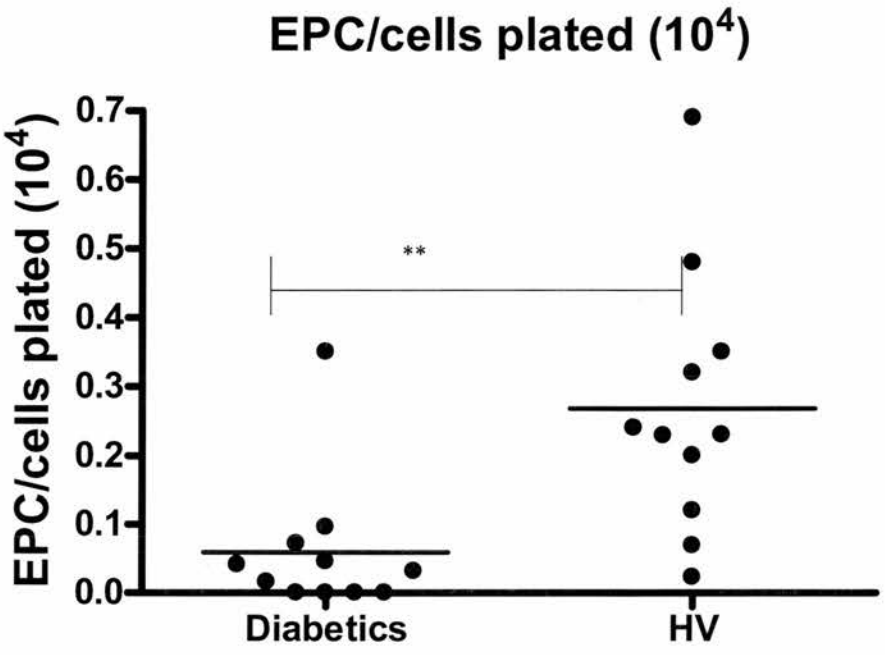


Figure 6.8 Comparison of median colony numbers and median percentage positivity of CFU-EPC in control group versus diabetic patient group.

The control group was not cohort matched with the patient group. The control group had a higher median number of cell colonies ($0.27x10^4 \pm 0.06x10^4$) compared with the patient group ($0.06x10^4 \pm 0.03x10^4$). This difference was statistically significant (p=0.004).

The patient group had a significantly lower median percentage of cells positive for CD34 compared to control group (Figure 6.9 and table 6.7).No other significant differences were found for the other markers studied (VEGFR2, VE-cadherin or for the cells co-exressing both CD34 and VEGFR2 markers)(table 6.7).

	Controls	Interquartile ranges	Patients	Interquartile ranges	p value
Number	37	-	45	-	-
Mean Age	40.9	-	37.4	-	0.24
CD34 mRNA RQ	3.660	1.8978.022	4.015	2.4526.518	0.5360
MVD+ MVD-	-	-	3.830 4.156	1.9735.491 3.0007.00	0.35
MVD-, HbA_{1c}>8 MVD-, HbA_{1c}<8	-	-	4.115 5.090	2.6405.997 3.2049.699	0.33
VEGFR2 mRNA RQ	1.302	0.5412.660	1.487	1.0182.603	0.3281
MVD+ MVD-	-	-	1.403 1.608	0.9052.805 1.0832.212	0.8773
MVD-, HbA_{1c}>8 MVD-, HbA_{1c}<8	-		0.8283 2.29	0.7391.201 1.4454.437	0.2571
VE-cadherin mRNA RQ	**2.919**	**1.5777.519**	**2.244**	**1.3733.256**	**0.0435**
MVD+ MVD-	-	-	2.930 1.997	1.8543.447 1.3692.773	0.1972
MVD-, HbA_{1c}>8 MVD-, HbA_{1c}<8	-	-	1.892 2.244	1.4992.421 1.0992.803	0.81
VWF mRNA RQ	2.170	1.2493.595	2.597	1.4744.229	0.18
MVD+ MVD-	-	-	2.230 3.149	2.0333.067 1.3034.805	0.48
MVD-, HbA_{1c}>8 MVD-, HbA_{1c}<8	-	-	2.091 3.596	1.3034.362 2.6024.946	0.42
Number	15	-	11 (cell culture) 21 (flow cytometry)		-
Mean Age	39.7	-	39.9 (cell culture) 34.7 (flow cytometry)		0.97 0.21
Median number of EPCs (per 10^6 cells)	**0.23**	**0.160.335**	**0.032**	**0-0.059**	**0.004**
Median %CD34+ cells	**0.06**	**0.050.09**	**0.02**	**0.020.05**	**0.0003**
Median %VEGFR2+ cells	1.89	0.8853.06	4	1.2132.33	0.14
Median %VE-cadherin+ cells	10.55	6.86513.11	12.6	8.3816.98	0.26
Median % CD34+VEGFR2+ cells	24.39	5.7337.345	15.08	0.22243.5	0.35

Table 6.7

Comparison between the patient group and control group of the number of EPCs detected by real-time PCR, flow cytometry and cell culture. Results are expressed as medians with interquartile ranges. The unpaired t-test was used to compare mean ages of patient and control groups. **MVD**: Microvascular disease. **HbA1c**: Haemoglobin A1c

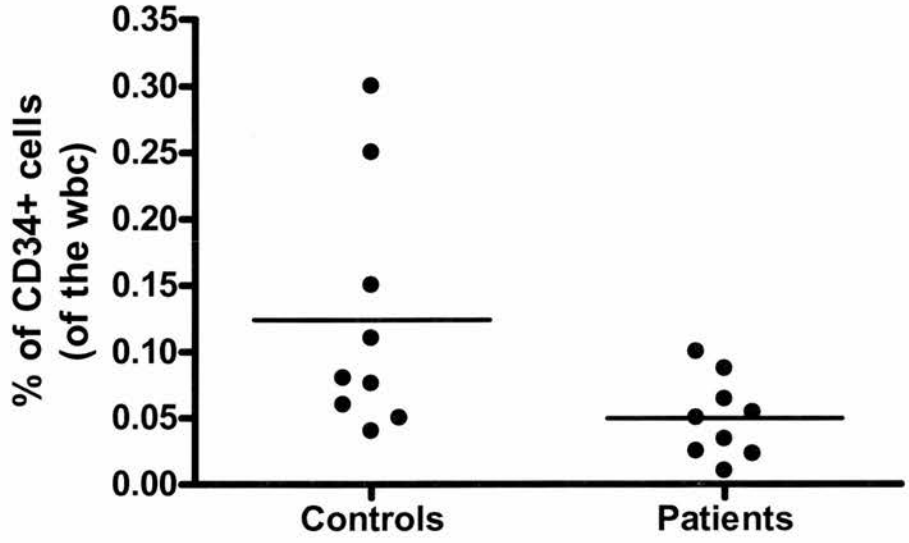


Figure 6.9

The percentage of cells expressing CD34 is significantly lower in the patient group compared to control group (p=0.003).

No significant differences were found in the median mRNA levels of CD34, VEGFR2 and vWF between patients with type 1-diabetes and control group (table 6.7). However, the patient group had a significantly lower median mRNA levels of VE-cadherin compared with the control group (Table 6.7) Furthermore, no differences were found in the mRNA levels in patients with or without microvascular disease or in patients with poorly controlled diabetes (Table 6.7).

6.3 Discussion

Circulating EPCs phenotyped by flow cytometry analysis and functional CFU-EPCs assessed by colony assay were measured in the different clinical situations and compared with healthy individual controls. The numbers of circulating endothelial progenitor cells have been reported to be reduced in individuals with ischaemic diseases (Hill *et al.*, 2003) for example in patients with cardiovascular risk factors (Vasa *et al.*, 2001) or in patients with diabetes mellitus type 2 (Tepper *et al.*, 2002).

Circulating endothelial progenitor cells in patients receiving elective surgery for abdominal aortic aneurysm repair and in type 1-diabetes patients selected as models of blood vessel damage and tissue ischaemia respectively were compared to a control group of healthy individuals. The mean number of functional CFU-EPCs in both clinical settings was significally reduced with comparison to that detected in the control group. However, the proportion and total numbers of $CD34^+$, $CD133^+$ and $VEGFR2^+$ cells alone or in combination did not differ significally in patients prior to aortic aneurysm repair or type 1-diabetes patients compared to control subjects with the exception of a significant reduction of $CD34^+$ cell numbers in type 1-diabetes patients compared to healthy individual controls. Circulating EPC numbers were reduced in patients with type 1-diabetes. In culture, blood derived $CD34^+$ cells from type 1-diabetes subjects produced fewer endothelial cells than those from non-diabetic controls (Schatteman *et al.*, 2000) which could be explained due to their lower number of CFU-EPC. This may have important consequences in terms of their ability to form collateral vessels as part of the vascular repair mechanism.

Supporting the data showed in Chapter 5 no correlation between the number of peripheral blood $CD34^+$ cells and the number of CFU-EPCs was found. Healthy individuals had higher number of CFU-EPCs compared to the patient groups but these effects were not associated with increases in circulating $CD34^+KDR^+$ phenotypic EPCs or leucocyte CD34 mRNA.

It is known that EPCs are mobilised from the bone marrow in response to vascular injury and tissue ischaemia and that this is cytokine driven. Previous studies have demonstrated early mobilisation of EPCs following vascular injury in patients with acute myocardial infarction (Massa *et al.*, 2005), major burns and following coronary artery bypass grafting (CABG)(Gill *et al.*, 2001). Acute myocardial infarction was associated with a rapid increase of EPCs in the circulation (Shintani *et al.*, 2001). These clinical events involve extensive damage to a number of tissues in addition to the vasculature, which may contribute to the mobilisation of progenitors. In this

Chapter we demonstrate that local selective vascular injury can also influence the number of circulating progenitor cells. Percutaneous coronary intervention, but not diagnostic angiography, was associated with a mild systemic inflammatory response and an increase in functional CFU-EPCs in the peripheral blood. Coronary intervention increased the number of CFU-EPC. However, these effects were not associated with increases in circulating $CD34^+KDR^+$ phenotypic EPCs or leucocyte CD34 mRNA. Therefore, again no correlation between the number of peripheral blood $CD34^+$ cells and the number of CFU-EPCs was found.

Gill *et al.*, (2001) suggested that vascular trauma induced a rapid but transient mobilisation of endothelial precursors. In this study, following elective abdominal aortic aneurysm repair patients showed a transient reduction in the mean number of CFU-EPC colonies by 24h after the procedure followed by an increase in CFU-EPC at 48h. This did not reach significance but further data needs to be collected to confirm this trend. A significant decrease in the proportion of $CD34^+$ and $CD133^+$ cells was also detected after 24h post-operatively, with recovery at 48h. In this case both flow cytometry, mRNA analysis and CFU-EPC colony assay correlated with each other. In addition to the flow cytometry analysis real-time PCR showed a rapid but transient reduction in the relative quantities of mRNA for CD34 VEGFR2 and CD133, with a subsequent increase in the relative expression of mRNA for VE-cadherin and vWf following aneurysm repair. Our studies demonstrate that there is mobilisation of a circulating pool of EPCs, which may reflect a rapid maturation process as part of the post-operative vascular repair mechanism.

The original description of the putative endothelial progenitor cell was based on cell culture and adhesion techniques. Asahara *et al.*, (1997). described a population of adult human circulating $CD34^+$ cells that could differentiate into cells with endothelial-like characteristics *in vitro*. The exact origin and phenotype of these progenitors remains a matter of debate in part because the purity of $CD34^+$ cells used in this initial study was only 15% Asahara *et al.*, 1997). Subsequently the co-expression of transmembrane glycoproteins CD34 and vascular endothelial growth factor receptor-2 (VEGFR-2 or KDR) have been used in an increasing number of clinical studies to phenotype and to quantify circulating EPCs. As the field has developed, an increasing number of methods have emerged to define vascular progenitors and quantify regenerative capacity. The CFU-EPC assay has been used widely since it was first described by Hill *et al.*, (2003). Whilst quantification of CFU-EPC provides a measure of the capacity of circulating mononuclear cells to form endothelial cells, it is doubtful whether these colonies arise directly from the circulating $CD34^+$ stem cells (see 5.2.3.4). Studies addressing the origin of endothelial

progenitor lineage in adult peripheral blood have demonstrated that monocytes also express endothelial lineage markers such as VEGFR-2 and can differentiate into mature endothelial cells (as presented in 5.1.6). Direct staining of our colonies demonstrated that CFU-EPCs not only behave like mature endothelial cells and incorporate acetylated LDL, but that they also strongly express CD14. In keeping with recent reports (as reviewed in 5.1.6), we have found that isolated $CD34^+$ cells do not give rise to spontaneous CFU-EPCs but die in this assay. In contrast, all CFU-EPC activity is found in $CD14^+$ enriched, but not in $CD14^+$ depleted, fractions of peripheral blood mononuclear cells (see 5.2.3.4). This population of circulating $CD14^+$ monocyte-like endothelial precursors appear more functionally mature than $CD34^+$ cells and are more abundant in normal peripheral blood than in bone marrow. In contrast, while $CD34^+KDR^+$ cells may ultimately give rise to endothelial cells, they are much less frequent in peripheral blood than in bone marrow, and may only be the precursors of the functionally more mature CFU-EPC cells that appear to be crucial for the immediate response to vascular injury.

Whilst in these clinical studies we did not specifically measure $CD14^+$ cells by flow cytometry, we did quantify monocyte numbers and total leucocyte CD14 mRNA. We identified a reproducible decrease in both immediately after cardiac catheterisation. It is possible that these cells immediately localize to the site of vessel damage: both at the site of arterial puncture and at the site of coronary angioplasty and stenting. The number of peripheral blood monocytes was restored to pre-procedural levels by 24 hours, and CD14 mRNA levels increased 24 hours after PCI. Mobilisation of $CD14^+$ monocyte-like cells may explain the increase in CFU-EPCs in peripheral blood observed 24 hours after arterial injury. These cells may contribute to vascular repair either through formation of mature endothelial cells and incorporation into the vessel wall, or through the release of angiogenic growth factors at the site of vessel injury. Further studies are required to explore specifically the role of $CD14^+$ cells in vascular injury and repair.

General conclusions

In the first part of this thesis (Chapter 3), we have developed a simplified method, compared to a range of complex published systems, to generate mature functional neutrophils from $CD34^+$ cells. Future clinical application as a possible means of reducing neutropenia following autologous peripheral blood stem cell transplantation, still not solved by availability of donor granulocyte transfusions, remains to be tested and cost effectiveness examined. Our results showed that adddition of thrombopoietin employed by other methods especially where joint neutrophil and maegakaryocyte expansion/maturation is intended, is not necessary for selective neutrophil expansion and it showed an inhibitory effect on neutrophil maturation. If megakaryocyte expansion is to be considered then separate protocols should be devised to optimise this in distinct cultures rather than attempting simultaneous neutrophil and megakaryocyte growth in the same system.In addition, this *in vitro* $CD34^+$ neutrophil differentiation system is being demonstrated in Chapter 4 as a good model to study changes in the expression of molecules, in our case phosphatases, that might occur through the neutrophil differentiation pathway. In the second part of this thesis we examined the endothelial progenitor potential of feasible sources of autologous EPCs, and we have completed a systematic study of a range of proposed phenotypes of EPC with regard to HSC markers. In addition, a CFU-EPC assay has also been tested in all the different EPC sources. No relationship between phenotype definition and CFU-EPC function has been found. $CD34^+$ cells from any source do not form colonies, do not take up typical spindle-shaped morphology, and die after a few days in the CFU-EPC. $CD14^+$ cells contain all CFU-EPC. When purified $CD34^+$ cells are expanded in the presence of certain growth factors and other cytokines they will survive, assume spindle-shaped morphology, and some will form colonies in the CFU-EPC assay. This implies that $CD34^+$ cells have the potential to develop into CFU-EPC capable cells. Therefore, precursors of the $CD14^+$ cells CFU-EPC exist within the HSC-rich sources. These CFU-EPC precursors may be the $CD34^+VEGFR2^+$ identified by others, and represent an earlier state of differentiation of the same lineage which gives rise to CFU-EPC and ultimately to mature endothelial cells incorporated in vessels. However, while $CD14^+$ give rise to endothelial-like cells it is still under investigation whether these $CD14^+$ EPC precursor are monocytes with a retained considerable proliferative potential, or a true independent EPC precursor resembling monocytes by expression

of CD14.

Using *in vitro* assays such as phenotype and CFU-EPC can not determine vasculogenic potential directly. In the next 3 years, models of vasculogenesis in SCID-NOD mice will be used to evaluate the potential of human mononuclear cells in the contribution to *de novo* angiogenesis and vascular repair *in vivo*. This may enable correlation of the *in vitro* assays with vasculogenic potential, or indicate alternative assays which better indicate vasculogenic potential. This study should then provide information on the relative angiogenic potential of available sources of endothelial progenitor cell for autologous clinical engraftment procedures envisaged for cardiac and critical limb ischaemia, and whether these can be improved by selective subpopulation enrichment of *ex vivo* endothelial promoting manipulation. These should also offer informed choices for design and implementation of trials of clinical procedures, especially where the angiogenic potential of such sources in target patient groups may be impaired.

References

Adachi K, Suzuki M, Sugimoto T, Uetsuka K, Nakamaya H, Doi K. Effects of granulocyte colony-stimulating factor on the kinetics of inflammatory cells in the peripheral blood and pulmonary lesions during the development of bleomycin-induced lung injury in rats. Exp Toxicol Pathol 2003; 55(1):21-32.

Adams V, Lenk K, Linke A, Lenz D, Erbs S, Sandri M et al. Increase of circulating endothelial progenitor cells in patients with coronary artery disease after exercise-induced ischemia. Arterioscler Thromb Vasc Biol 2004; 24(4):684-690.

Ahmet I, Sawa Y, Yamaguchi T, Matsuda H. Gene transfer of hepatocyte growth factor improves angiogenesis and function of chronic ischemic myocardium in canine heart. Ann Thorac Surg 2003; 75(4):1283-1287.

Aicher A, Zeiher AM, Dimmeler S. Mobilizing endothelial progenitor cells. Hypertension 2005; 45(3):321-325.

Alessandri G, Emanueli C, Madeddu P. Genetically engineered stem cell therapy for tissue regeneration. Ann N Y Acad Sci 2004; 1015:271-284.

Amit M, Carpenter MK, Inokuma MS, Chiu CP, Harris CP, Waknitz MA et al. Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. Dev Biol 2000; 227(2):271-278.

Andrews RG, Singer JW, Bernstein ID. Monoclonal antibody 12-8 recognizes a 115-kd molecule present on both unipotent and multipotent hematopoietic colony-forming cells and their precursors. Blood 1986; 67(3):842-845.

Angoulvant D, Clerc A, Benchalal S, Galambrun C, Farre A, Bertrand Y et al. Human mesenchymal stem cells suppress induction of cytotoxic response to alloantigens. Biorheology 2004; 41(3-4):469-476.

Angst BD, Marcozzi C, Magee AI. The cadherin superfamily: diversity in form and function. J Cell Sci 2001; 114(Pt 4):629-641.

Annex BH, Simons M. Growth factor-induced therapeutic angiogenesis in the heart: protein therapy. Cardiovasc Res 2005; 65(3):649-655.

Asahara T (a), Masuda H, Takahashi T, Kalka C, Pastore C, Silver M et al. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circ Res 1999; 85(3):221-228.

Asahara T (b), Takahashi T, Masuda H, Kalka C, Chen D, Iwaguro H et al. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. EMBO J 1999; 18(14):3964-3972.

Asahara T, Murohara T, Sullivan A, Silver M, van der ZR, Li T et al. Isolation of putative

progenitor endothelial cells for angiogenesis. Science 1997; 275(5302):964-967.

Asahara T, Kawamoto A. Endothelial progenitor cells for postnatal vasculogenesis. Am J Physiol Cell Physiol 2004; 287(3):C572-C579.

Assmus B, Schachinger V, Teupe C, Britten M, Lehmann R, Dobert N et al. Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI). Circulation 2002; 106(24):3009-3017.

Autiero M, Luttun A, Tjwa M, Carmeliet P. Placental growth factor and its receptor, vascular endothelial growth factor receptor-1: novel targets for stimulation of ischemic tissue revascularization and inhibition of angiogenic and inflammatory disorders. J Thromb Haemost 2003; 1(7):1356-1370.

Awad O, Jiao C, Ma N, Dunnwald M, Schatteman GC. Obese diabetic mouse environment differentially affects primitive and monocytic endothelial cell progenitors. Stem Cells 2005; 23(4):575-583.

Bahlmann FH, De Groot K, Spandau JM, Landry AL, Hertel B, Duckert T et al. Erythropoietin regulates endothelial progenitor cells. Blood 2004; 103(3):921-926.

Bailey AS, Fleming WH. Converging roads: evidence for an adult hemangioblast. Exp Hematol 2003; 31(11):987-993.

Bailey AS, Jiang S, Afentoulis M, Baumann CI, Schroeder DA, Olson SB et al. Transplanted adult hematopoietic stems cells differentiate into functional endothelial cells. Blood 2004; 103(1):13-19.

Barrett JA. An introduction to bone marrow transplantation and processing. In: Gee AP, editor. Bone Marrow Processing and Purging. Boca Raton, FL: CRC Press, 1991.

Barry F, Boynton R, Murphy M, Haynesworth S, Zaia J. The SH-3 and SH-4 antibodies recognize distinct epitopes on CD73 from human mesenchymal stem cells. Biochem Biophys Res Commun 2001; 289(2):519-524.

Bartholomew A, Sturgeon C, Siatskas M, Ferrer K, McIntosh K, Patil S et al. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. Exp Hematol 2002; 30(1):42-48.

Beekhuizen H, Blokland I, Corsel-Van Tilburg AJ, Koning F, Van Furth R. CD14 contributes to the adherence of human monocytes to cytokine-stimulated endothelial cells. J Immunol 1991; 147(11):3761-3767.

Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S et al. Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell 2003; 114(6):763-776.

Bender JG SSaUK. Ex vivo expansion of Neutrophil precursors form Blood CD34+ cells: Applications for the treatment of Neutropenia. Hematopoietic Stem Cells. NY: 1995: 171.

Berenson RJ et al. Selection and Transplantation of Autologous Hematopoietic Progenitor cells. In: Levitt D MR, editor. Hematopoietic Stem Cells. NY: 1995: 519.

Bergsten E, Uutela M, Li X, Pietras K, Ostman A, Heldin CH et al. PDGF-D is a specific, protease-activated ligand for the PDGF beta-receptor. Nat Cell Biol 2001; 3(5):512-516.

Bertolini F, Battaglia M, Pedrazzoli P, Da Prada GA, Lanza A, Soligo D et al. Megakaryocytic progenitors can be generated ex vivo and safely administered to autologous peripheral blood progenitor cell transplant recipients. Blood 1997; 89(8):2679-2688.

Bhattacharya V, McSweeney PA, Shi Q, Bruno B, Ishida A, Nash R et al. Enhanced endothelialization and microvessel formation in polyester grafts seeded with CD34(+) bone marrow cells. Blood 2000; 95(2):581-585.

Blann AD, Woywodt A, Bertolini F, Bull TM, Buyon JP, Clancy RM et al. Circulating endothelial cells. Biomarker of vascular disease. Thromb Haemost 2005; 93(2):228-235.

Blann AD. Plasma von Willebrand factor, thrombosis, and the endothelium: the first 30 years. Thromb Haemost 2006; 95(1):49-55.

Bonnet D. Haematopoietic stem cells. J Pathol 2002; 197(4):430-440.

Botta R, Gao E, Stassi G, Bonci D, Pelosi E, Zwas D et al. Heart infarct in NOD-SCID mice: therapeutic vasculogenesis by transplantation of human CD34+ cells and low dose CD34+KDR+ cells. FASEB J 2004; 18(12):1392-1394.

Bouis D, Hospers GA, Meijer C, Molema G, Mulder NH. Endothelium in vitro: a review of human vascular endothelial cell lines for blood vessel-related research. Angiogenesis 2001; 4(2):91-102.

Boyer M, Townsend LE, Vogel LM, Falk J, Reitz-Vick D, Trevor KT et al. Isolation of endothelial cells and their progenitor cells from human peripheral blood. J Vasc Surg 2000; 31(1 Pt 1):181-189.

Briones MA, Josephson CD, Hillyer CD. Granulocyte transfusion: revisited. Curr Hematol Rep 2003; 2(6):522-527.

Britten MB, Abolmaali ND, Assmus B, Lehmann R, Honold J, Schmitt J et al. Infarct remodeling after intracoronary progenitor cell treatment in patients with acute myocardial infarction (TOPCARE-AMI): mechanistic insights from serial contrast-enhanced magnetic resonance imaging. Circulation 2003; 108(18):2212-2218.

Brockes JP. Amphibian limb regeneration: rebuilding a complex structure. Science 1997; 276(5309):81-87.

Brown KA, Vora A, Biggerstaff J, Edgell CJ, Oikle S, Mazure G et al. Application of an immortalized human endothelial cell line to the leucocyte:endothelial adherence assay. J Immunol Methods 1993; 163(1):13-22.

Brugger W, Mocklin W, Heimfeld S, Berenson RJ, Mertelsmann R, Kanz L. Ex vivo expansion of enriched peripheral blood CD34+ progenitor cells by stem cell factor, interleukin-1 beta (IL-1 beta), IL-6, IL-3, interferon-gamma, and erythropoietin. Blood 1993; 81(10):2579-2584.

Cairo MS, Wagner JE. Placental and/or umbilical cord blood: an alternative source of

hematopoietic stem cells for transplantation. Blood 1997; 90(12):4665-4678.

Calera MR, Venkatakrishnan A, Kazlauskas A. VE-cadherin increases the half-life of VEGF receptor 2. Exp Cell Res 2004; 300(1):248-256.

Calvi LM, Adams GB, Weibrecht KW, Weber JM, Olson DP, Knight MC et al. Osteoblastic cells regulate the haematopoietic stem cell niche. Nature 2003; 425(6960):841-846.

Camargo FD, Green R, Capetanaki Y, Jackson KA, Goodell MA. Single hematopoietic stem cells generate skeletal muscle through myeloid intermediates. Nat Med 2003; 9(12):1520-1527.

Cao R, Brakenhielm E, Li X, Pietras K, Widenfalk J, Ostman A et al. Angiogenesis stimulated by PDGF-CC, a novel member in the PDGF family, involves activation of PDGFR-alphaalpha and -alphabeta receptors. FASEB J 2002; 16(12):1575-1583.

Cao Y, Linden P, Farnebo J, Cao R, Eriksson A, Kumar V et al. Vascular endothelial growth factor C induces angiogenesis in vivo. Proc Natl Acad Sci U S A 1998; 95(24):14389-14394.

Cao Y. Tumor angiogenesis and therapy. Biomed Pharmacother 2005; 59 Suppl 2:S340-S343.

Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M et al. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 1996; 380(6573):435-439.

Carmeliet P, Lampugnani MG, Moons L, Breviario F, Compernolle V, Bono F et al. Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. Cell 1999; 98(2):147-157.

Carmeliet P. VEGF gene therapy: stimulating angiogenesis or angioma-genesis? Nat Med 2000; 6(10):1102-1103.

Casteilla L, Planat-Benard V, Cousin B, Silvestre JS, Laharrague P, Charriere G et al. Plasticity of adipose tissue: a promising therapeutic avenue in the treatment of cardiovascular and blood diseases? Arch Mal Coeur Vaiss 2005; 98(9):922-926.

Chan RJ, Johnson SA, Li Y, Yoder MC, Feng GS. A definitive role of Shp-2 tyrosine phosphatase in mediating embryonic stem cell differentiation and hematopoiesis. Blood 2003; 102(6):2074-2080.

Chao NJ, Blume KG. Bone marrow transplantation. Part I—Allogeneic. West J Med 1989; 151(6):638-643.

Chao NJ, Blume KG. Bone marrow transplantation. Part II—autologous. West J Med 1990; 152(1):46-51.

Cho HJ, Kim HS, Lee MM, Kim DH, Yang HJ, Hur J et al. Mobilized endothelial progenitor cells by granulocyte-macrophage colony-stimulating factor accelerate reendothelialization and reduce vascular inflammation after intravascular radiation. Circulation 2003; 108(23):2918-2925.

Choi K. The hemangioblast: a common progenitor of hematopoietic and endothelial cells. J

Hematother Stem Cell Res 2002; 11(1):91-101.

Chute JPa, Fung J, Muramoto G, Erwin R. Ex vivo culture rescues hematopoietic stem cells with long-term repopulating capacity following harvest from lethally irradiated mice. Exp Hematol 2004; 32(3):308-317.

Chute JPb, Muramoto G, Fung J, Oxford C. Quantitative analysis demonstrates expansion of SCID-repopulating cells and increased engraftment capacity in human cord blood following ex vivo culture with human brain endothelial cells. Stem Cells 2004; 22(2):202-215.

Cines DB, Pollak ES, Buck CA, Loscalzo J, Zimmerman GA, McEver RP et al. Endothelial cells in physiology and in the pathophysiology of vascular disorders. Blood 1998; 91(10):3527-3561.

Clanchy FI, Holloway AC, Lari R, Cameron PU, Hamilton JA. Detection and properties of the human proliferative monocyte subpopulation. J Leukoc Biol 2006; 79(4):757-766.

Cogle CR, Scott EW. The hemangioblast: cradle to clinic. Exp Hematol 2004; 32(10):885-890.

Conget PA, Minguell JJ. Phenotypical and functional properties of human bone marrow mesenchymal progenitor cells. J Cell Physiol 1999; 181(1):67-73.

Conrad C, Huss R. Adult stem cell lines in regenerative medicine and reconstructive surgery. J Surg Res 2005; 124(2):201-208.

Davani S, Deschaseaux F, Chalmers D, Tiberghien P, Kantelip JP. Can stem cells mend a broken heart? Cardiovasc Res 2005; 65(2):305-316.

De Bruyn C, Delforge A, Bernier M, Bron D. Ex vivo expansion of neutrophil precursor cells from fresh and cryopreserved cord blood cells. Cytotherapy 2003; 5(1):87-98.

de Wynter EA, Buck D, Hart C, Heywood R, Coutinho LH, Clayton A et al. CD34+AC133+ cells isolated from cord blood are highly enriched in long-term culture-initiating cells, NOD/SCID-repopulating cells and dendritic cell progenitors. Stem Cells 1998; 16(6):387-396.

Deans RJ, Moseley AB. Mesenchymal stem cells: biology and potential clinical uses. Exp Hematol 2000; 28(8):875-884.

Decaudin D, Vantelon JM, Bourhis JH, Farace F, Bonnet ML, Guillier M et al. Ex vivo expansion of megakaryocyte precursor cells in autologous stem cell transplantation for relapsed malignant lymphoma. Bone Marrow Transplant 2004; 34(12):1089-1093.

Del Papa N, Colombo G, Fracchiolla N, Moronetti LM, Ingegnoli F, Maglione W et al. Circulating endothelial cells as a marker of ongoing vascular disease in systemic sclerosis. Arthritis Rheum 2004; 50(4):1296-1304.

Delafontaine P, Song YH, Li Y. Expression, regulation, and function of IGF-1, IGF-1R, and IGF-1 binding proteins in blood vessels. Arterioscler Thromb Vasc Biol 2004; 24(3):435-444.

Di Cristofano A, Pandolfi PP. The multiple roles of PTEN in tumor suppression. Cell 2000; 100(4):387-390.

Di Nicola M, Carlo-Stella C, Magni M, Milanesi M, Longoni PD, Matteucci P et al. Human bone

marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood 2002; 99(10):3838-3843.

Dieterlen-Lievre F, Pardanaud L, Bollerot K, Jaffredo T. Hemangioblasts and hemopoietic stem cells during ontogeny. C R Biol 2002; 325(10):1013-1020.

Dignat-George F, Sampol J. Circulating endothelial cells in vascular disorders: new insights into an old concept. Eur J Haematol 2000; 65(4):215-220.

Dimmeler S, Aicher A, Vasa M, Mildner-Rihm C, Adler K, Tiemann M et al. HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI 3-kinase/Akt pathway. J Clin Invest 2001; 108(3):391-397.

Donnelly DS, Zelterman D, Sharkis S, Krause DS. Functional activity of murine CD34+ and. Exp Hematol 1999; 27(5):788-796.

Elsheikh E, Uzunel M, He Z, Holgersson J, Nowak G, Sumitran-Holgersson S. Only a specific subset of human peripheral-blood monocytes has endothelial-like functional capacity. Blood 2005; 106(7):2347-2355.

Emeis JJ, Edgell CJ. Fibrinolytic properties of a human endothelial hybrid cell line (Ea.hy 926). Blood 1988; 71(6):1669-1675.

Esser S, Lampugnani MG, Corada M, Dejana E, Risau W. Vascular endothelial growth factor induces VE-cadherin tyrosine phosphorylation in endothelial cells. J Cell Sci 1998; 111 (Pt 13):1853-1865.

Fadini GP, Miorin M, Facco M, Bonamico S, Baesso I, Grego F et al. Circulating endothelial progenitor cells are reduced in peripheral vascular complications of type 2 diabetes mellitus. J Am Coll Cardiol 2005; 45(9):1449-1457.

Fan CL, Li Y, Gao PJ, Liu JJ, Zhang XJ, Zhu DL. Differentiation of endothelial progenitor cells from human umbilical cord blood CD 34+ cells in vitro. Acta Pharmacol Sin 2003; 24(3):212-218.

Fernandes KJ, McKenzie IA, Mill P, Smith KM, Akhavan M, Barnabe-Heider F et al. A dermal niche for multipotent adult skin-derived precursor cells. Nat Cell Biol 2004; 6(11):1082-1093.

Fernandez PB, Lucibello FC, Gehling UM, Lindemann K, Weidner N, Zuzarte ML et al. Endothelial-like cells derived from human CD14 positive monocytes. Differentiation 2000; 65(5):287-300.

Ferrara N, Bunting S. Vascular endothelial growth factor, a specific regulator of angiogenesis. Curr Opin Nephrol Hypertens 1996; 5(1):35-44.

Folkman J. Role of angiogenesis in tumor growth and metastasis. Semin Oncol 2002; 29(6 Suppl 16):15-18.

Forbes SJ, Vig P, Poulsom R, Wright NA, Alison MR. Adult stem cell plasticity: new pathways of tissue regeneration become visible. Clin Sci (Lond) 2002; 103(4):355-369.

Friedenstein AJ. Precursor cells of mechanocytes. Int Rev Cytol 1976; 47:327-359.

Fuchs S, Satler LF, Kornowski R, Okubagzi P, Weisz G, Baffour R et al. Catheter-based autologous bone marrow myocardial injection in no-option patients with advanced coronary artery disease: a feasibility study. J Am Coll Cardiol 2003; 41(10):1721-1724.

Fujiyama S, Amano K, Uehira K, Yoshida M, Nishiwaki Y, Nozawa Y et al. Bone marrow monocyte lineage cells adhere on injured endothelium in a monocyte chemoattractant protein-1-dependent manner and accelerate reendothelialization as endothelial progenitor cells. Circ Res 2003; 93(10):980-989.

Fulkes SM et al. Stem Cell Technologies Inc. 2003.

Galli R, Borello U, Gritti A, Minasi MG, Bjornson C, Coletta M et al. Skeletal myogenic potential of human and mouse neural stem cells. Nat Neurosci 2000; 3(10):986-991.

Gehling UM, Ergun S, Schumacher U, Wagener C, Pantel K, Otte M et al. In vitro differentiation of endothelial cells from AC133-positive progenitor cells. Blood 2000; 95(10):3106-3112.

Geissler K, Yin JA, Ganser A, Sanz MA, Szer J, Raghavachar A et al. Prior and concurrent administration of recombinant human megakaryocyte growth and development factor in patients receiving consolidation chemotherapy for de novo acute myeloid leukemia—a randomized, placebo-controlled, double-blind safety and efficacy study. Ann Hematol 2003; 82(11):677-683.

George J, Herz I, Goldstein E, Abashidze S, Deutch V, Finkelstein A et al. Number and adhesive properties of circulating endothelial progenitor cells in patients with in-stent restenosis. Arterioscler Thromb Vasc Biol 2003; 23(12):e57-e60.

Gill M, Dias S, Hattori K, Rivera ML, Hicklin D, Witte L et al. Vascular trauma induces rapid but transient mobilization of VEGFR2(+)AC133(+) endothelial precursor cells. Circ Res 2001; 88(2):167-174.

Giordano R, Lazzari L, Rebulla P. Clinical grade cell manipulation. Vox Sang 2004; 87(2):65-72.

Gojo S, Gojo N, Takeda Y, Mori T, Abe H, Kyo S et al. In vivo cardiovasculogenesis by direct injection of isolated adult mesenchymal stem cells. Exp Cell Res 2003; 288(1):51-59.

Goldman JM, Catovsky D, Hows J, Spiers AS, Galton DA. Cryopreserved peripheral blood cells functioning as autografts in patients with chronic granulocytic leukaemia in transformation. Br Med J 1979; 1(6174):1310-1313.

Good RA. Cellular immunology in a historical perspective. Immunol Rev 2002; 185:136-158.

Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med 1996; 183(4):1797-1806.

Goodell MA, Rosenzweig M, Kim H, Marks DF, DeMaria M, Paradis G et al. Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. Nat Med 1997; 3(12):1337-1345.

Graf L, Heimfeld S, Torok-Storb B. Comparison of gene expression in CD34+ cells from bone marrow and G-CSF-mobilized peripheral blood by high-density oligonucleotide array analysis. Biol Blood Marrow Transplant 2001; 7(9):486-494.

Grant MB, May WS, Caballero S, Brown GA, Guthrie SM, Mames RN et al. Adult hematopoietic stem cells provide functional hemangioblast activity during retinal neovascularization. Nat Med 2002; 8(6):607-612.

Gregory CA, Prockop DJ, Spees JL. Non-hematopoietic bone marrow stem cells: molecular control of expansion and differentiation. Exp Cell Res 2005; 306(2):330-335.

Gulati R, Jevremovic D, Peterson TE, Chatterjee S, Shah V, Vile RG et al. Diverse origin and function of cells with endothelial phenotype obtained from adult human blood. Circ Res 2003; 93(11):1023-1025.

Gulati R, Simari RD. Autologous cell-based therapies for vascular disease. Trends Cardiovasc Med 2004; 14(7):262-267.

Guo H, Fang B, Liao L, Zhao Z, Liu J, Chen H et al. Hemangioblastic characteristics of fetal bone marrow-derived Flk1(+)CD31(-)CD34(-) cells. Exp Hematol 2003; 31(7):650-658.

Hailman E, Lichenstein HS, Wurfel MM, Miller DS, Johnson DA, Kelley M et al. Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14. J Exp Med 1994; 179(1):269-277.

Hanahan D, Christofori G, Naik P, Arbeit J. Transgenic mouse models of tumour angiogenesis: the angiogenic switch, its molecular controls, and prospects for preclinical therapeutic models. Eur J Cancer 1996; 32A(14):2386-2393.

Harraz M, Jiao C, Hanlon HD, Hartley RS, Schatteman GC. CD34- Blood-Derived Human Endothelial Cell Progenitors. Stem Cells 2001; 19(4):304-312.

Hartmann O, Le Corroller AG, Blaise D, Michon J, Philip I, Norol F et al. Peripheral blood stem cell and bone marrow transplantation for solid tumors and lymphomas: hematologic recovery and costs. A randomized, controlled trial. Ann Intern Med 1997; 126(8):600-607.

Hattori K (a), Dias S, Heissig B, Hackett NR, Lyden D, Tateno M et al. Vascular endothelial growth factor and angiopoietin-1 stimulate postnatal hematopoiesis by recruitment of vasculogenic and hematopoietic stem cells. J Exp Med 2001; 193(9):1005-1014.

Hattori K (b), Heissig B, Tashiro K, Honjo T, Tateno M, Shieh JH et al. Plasma elevation of stromal cell-derived factor-1 induces mobilization of mature and immature hematopoietic progenitor and stem cells. Blood 2001; 97(11):3354-3360.

Haylock DN, To LB, Dowse TL, Juttner CA, Simmons PJ. Ex vivo expansion and maturation of peripheral blood CD34+ cells into the myeloid lineage. Blood 1992; 80(6):1405-1412.

Heeschen C, Aicher A, Lehmann R, Fichtlscherer S, Vasa M, Urbich C et al. Erythropoietin is a potent physiologic stimulus for endothelial progenitor cell mobilization. Blood 2003; 102(4):1340-1346.

Heike T, Nakahata T. Ex vivo expansion of hematopoietic stem cells by cytokines. Biochim Biophys Acta 2002; 1592(3):313-321.

Herbrig K, Haensel S, Oelschlaegel U, Pistrosch F, Foerster S, Passauer J. Endothelial dysfunction in patients with rheumatoid arthritis is associated with a reduced number and impaired function of endothelial progenitor cells. Ann Rheum Dis 2006; 65(2):157-163.

Higashi Y, Kimura M, Hara K, Noma K, Jitsuiki D, Nakagawa K et al. Autologous bone-marrow mononuclear cell implantation improves endothelium-dependent vasodilation in patients with limb ischemia. Circulation 2004; 109(10):1215-1218.

Hill JM, Zalos G, Halcox JP, Schenke WH, Waclawiw MA, Quyyumi AA et al. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. N Engl J Med 2003; 348(7):593-600.

Hino M, Suzuki K, Yamane T, Sakai N, Kubota H, Koh KR et al. Ex vivo expansion of mature human neutrophils with normal functions from purified peripheral blood CD34+ haematopoietic progenitor cells. Br J Haematol 2000; 109(2):314-321.

Hirschi KK, Rohovsky SA, Beck LH, Smith SR, D'Amore PA. Endothelial cells modulate the proliferation of mural cell precursors via platelet-derived growth factor-BB and heterotypic cell contact. Circ Res 1999; 84(3):298-305.

Hofmann M, Wollert KC, Meyer GP, Menke A, Arseniev L, Hertenstein B et al. Monitoring of bone marrow cell homing into the infarcted human myocardium. Circulation 2005; 111(17):2198-2202.

Holash J, Wiegand SJ, Yancopoulos GD. New model of tumor angiogenesis: dynamic balance between vessel regression and growth mediated by angiopoietins and VEGF. Oncogene 1999; 18(38):5356-5362.

Holyoake TL, Alcorn MJ, Richmond L, Farrell E, Pearson C, Green R et al. CD34 positive PBPC expanded ex vivo may not provide durable engraftment following myeloablative chemoradiotherapy regimens. Bone Marrow Transplant 1997; 19(11):1095-1101.

Hristov M (a), Erl W, Weber PC. Endothelial progenitor cells: isolation and characterization. Trends Cardiovasc Med 2003; 13(5):201-206.

Hristov M (b), Erl W, Weber PC. Endothelial progenitor cells: mobilization, differentiation, and homing. Arterioscler Thromb Vasc Biol 2003; 23(7):1185-1189.

Hunting CB, Noort WA, Zwaginga JJ. Circulating endothelial (progenitor) cells reflect the state of the endothelium: vascular injury, repair and neovascularization. Vox Sang 2005; 88(1):1-9.

Hur J, Yoon CH, Kim HS, Choi JH, Kang HJ, Hwang KK et al. Characterization of two types of endothelial progenitor cells and their different contributions to neovasculogenesis. Arterioscler Thromb Vasc Biol 2004; 24(2):288-293.

Ince H, Petzsch M, Kleine HD, Eckard H, Rehders T, Burska D et al. Prevention of left ventricular

remodeling with granulocyte colony-stimulating factor after acute myocardial infarction: final 1-year results of the Front-Integrated Revascularization and Stem Cell Liberation in Evolving Acute Myocardial Infarction by Granulocyte Colony-Stimulating Factor (FIRSTLINE-AMI) Trial. Circulation 2005; 112(9 Suppl):I73-I80.

Ingram DA, Mead LE, Tanaka H, Meade V, Fenoglio A, Mortell K et al. Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. Blood 2004; 104(9):2752-2760.

Ingram DA, Caplice NM, Yoder MC. Unresolved questions, changing definitions, and novel paradigms for defining endothelial progenitor cells. Blood 2005; 106(5):1525-1531.

Ishizawa K, Kubo H, Yamada M, Kobayashi S, Suzuki T, Mizuno S et al. Hepatocyte growth factor induces angiogenesis in injured lungs through mobilizing endothelial progenitor cells. Biochem Biophys Res Commun 2004; 324(1):276-280.

Iwaguro H, Yamaguchi J, Kalka C, Murasawa S, Masuda H, Hayashi S et al. Endothelial progenitor cell vascular endothelial growth factor gene transfer for vascular regeneration. Circulation 2002; 105(6):732-738.

Iwakura A, Luedemann C, Shastry S, Hanley A, Kearney M, Aikawa R et al. Estrogen-mediated, endothelial nitric oxide synthase-dependent mobilization of bone marrow-derived endothelial progenitor cells contributes to reendothelialization after arterial injury. Circulation 2003; 108(25):3115-3121.

Iwase T, Nagaya N, Fujii T, Itoh T, Murakami S, Matsumoto T et al. Comparison of angiogenic potency between mesenchymal stem cells and mononuclear cells in a rat model of hindlimb ischemia. Cardiovasc Res 2005; 66(3):543-551.

Jackson KA, Majka SM, Wang H, Pocius J, Hartley CJ, Majesky MW et al. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. J Clin Invest 2001; 107(11):1395-1402.

Jaffe EA, Nachman RL, Becker CG, Minick CR. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J Clin Invest 1973; 52(11):2745-2756.

Jaroscak J, Goltry K, Smith A, Waters-Pick B, Martin PL, Driscoll TA et al. Augmentation of umbilical cord blood (UCB) transplantation with ex vivo-expanded UCB cells: results of a phase 1 trial using the AastromReplicell System. Blood 2003; 101(12):5061-5067.

Jersmann HP, Hii CS, Hodge GL, Ferrante A. Synthesis and surface expression of CD14 by human endothelial cells. Infect Immun 2001; 69(1):479-485.

Jiang Y, Vaessen B, Lenvik T, Blackstad M, Reyes M, Verfaillie CM. Multipotent progenitor cells can be isolated from postnatal murine bone marrow, muscle, and brain. Exp Hematol 2002; 30(8):896-904.

Kalka C (a), Masuda H, Takahashi T, Kalka-Moll WM, Silver M, Kearney M et al. Transplantation

of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. Proc Natl Acad Sci U S A 2000; 97(7):3422-3427.

Kalka C (b), Masuda H, Takahashi T, Gordon R, Tepper O, Gravereaux E et al. Vascular endothelial growth factor(165) gene transfer augments circulating endothelial progenitor cells in human subjects. Circ Res 2000; 86(12):1198-1202.

Kalka C (c), Tehrani H, Laudenberg B, Vale PR, Isner JM, Asahara T et al. VEGF gene transfer mobilizes endothelial progenitor cells in patients with inoperable coronary disease. Ann Thorac Surg 2000; 70(3):829-834.

Kang HJ, Kim HS, Zhang SY, Park KW, Cho HJ, Koo BK et al. Effects of intracoronary infusion of peripheral blood stem-cells mobilised with granulocyte-colony stimulating factor on left ventricular systolic function and restenosis after coronary stenting in myocardial infarction: the MAGIC cell randomised clinical trial. Lancet 2004; 363(9411):751-756.

Kasai M, Kiyama Y, Kawamura A. Application of peripheral blood stem cells (PBSC) mobilized by recombinant human granulocyte colony stimulating factor for allogeneic PBSC transplantation and the comparison of allogeneic PBSC transplantation and bone marrow transplantation. Transfus Apher Sci 2002; 26(2):121-127.

Kaushal S, Amiel GE, Guleserian KJ, Shapira OM, Perry T, Sutherland FW et al. Functional small-diameter neovessels created using endothelial progenitor cells expanded ex vivo. Nat Med 2001; 7(9):1035-1040.

Kawamoto A, Gwon HC, Iwaguro H, Yamaguchi JI, Uchida S, Masuda H et al. Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia. Circulation 2001; 103(5):634-637.

Kawamoto A, Tkebuchava T, Yamaguchi J, Nishimura H, Yoon YS, Milliken C et al. Intramyocardial transplantation of autologous endothelial progenitor cells for therapeutic neovascularization of myocardial ischemia. Circulation 2003; 107(3):461-468.

Kawamura A, Horie T, Tsuda I, Ikeda A, Egawa H, Imamura E et al. Prevention of limb amputation in patients with limbs ulcers by autologous peripheral blood mononuclear cell implantation. Ther Apher Dial 2005; 9(1):59-63.

Kelly BD, Hackett SF, Hirota K, Oshima Y, Cai Z, Berg-Dixon S et al. Cell type-specific regulation of angiogenic growth factor gene expression and induction of angiogenesis in nonischemic tissue by a constitutively active form of hypoxia-inducible factor 1. Circ Res 2003; 93(11):1074-1081.

Kennedy M, Firpo M, Choi K, Wall C, Robertson S, Kabrun N et al. A common precursor for primitive erythropoiesis and definitive haematopoiesis. Nature 1997; 386(6624):488-493.

Kocher AA, Schuster MD, Szabolcs MJ, Takuma S, Burkhoff D, Wang J et al. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. Nat Med

2001; 7(4):430-436.

Kodama H, Inoue T, Watanabe R, Yasutomi D, Kawakami Y, Ogawa S et al. Neurogenic potential of progenitors derived from human circulating CD14+ monocytes. Immunol Cell Biol 2006; 84(2):209-217.

Kong D, Melo LG, Gnecchi M, Zhang L, Mostoslavsky G, Liew CC et al. Cytokine-induced mobilization of circulating endothelial progenitor cells enhances repair of injured arteries. Circulation 2004; 110(14):2039-2046.

Kortesidis A, Zannettino A, Isenmann S, Shi S, Lapidot T, Gronthos S. Stromal-derived factor-1 promotes the growth, survival, and development of human bone marrow stromal stem cells. Blood 2005; 105(10):3793-3801.

Krampera M, Glennie S, Dyson J, Scott D, Laylor R, Simpson E et al. Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. Blood 2003; 101(9):3722-3729.

Krause DS, Theise ND, Collector MI, Henegariu O, Hwang S, Gardner R et al. Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. Cell 2001; 105(3):369-377.

Kureishi Y, Luo Z, Shiojima I, Bialik A, Fulton D, Lefer DJ et al. The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animals. Nat Med 2000; 6(9):1004-1010.

Kusunoki T, Hailman E, Juan TS, Lichenstein HS, Wright SD. Molecules from Staphylococcus aureus that bind CD14 and stimulate innate immune responses. J Exp Med 1995; 182(6):1673-1682.

Kuwana M, Okazaki Y, Kodama H, Izumi K, Yasuoka H, Ogawa Y et al. Human circulating CD14+ monocytes as a source of progenitors that exhibit mesenchymal cell differentiation. J Leukoc Biol 2003; 74(5):833-845.

Lakshmipathy U, Verfaillie C. Stem cell plasticity. Blood Rev 2005; 19(1):29-38.

Lanza F, Healy L, Sutherland DR. Structural and functional features of the CD34 antigen: an update. J Biol Regul Homeost Agents 2001; 15(1):1-13.

Lapidot T, Petit I. Current understanding of stem cell mobilization: the roles of chemokines, proteolytic enzymes, adhesion molecules, cytokines, and stromal cells. Exp Hematol 2002; 30(9):973-981.

Laslett AL, Filipczyk AA, Pera MF. Characterization and culture of human embryonic stem cells. Trends Cardiovasc Med 2003; 13(7):295-301.

Laufs U, Werner N, Link A, Endres M, Wassmann S, Jurgens K et al. Physical training increases endothelial progenitor cells, inhibits neointima formation, and enhances angiogenesis. Circulation 2004; 109(2):220-226.

Le Blanc K, Tammik L, Sundberg B, Haynesworth SE, Ringden O. Mesenchymal stem cells

inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. Scand J Immunol 2003; 57(1):11-20.

Le Blanc K, Pittenger M. Mesenchymal stem cells: progress toward promise. Cytotherapy 2005; 7(1):36-45.

Lee RJ, Springer ML, Blanco-Bose WE, Shaw R, Ursell PC, Blau HM. VEGF gene delivery to myocardium: deleterious effects of unregulated expression. Circulation 2000; 102(8):898-901.

Lee SH, Wolf PL, Escudero R, Deutsch R, Jamieson SW, Thistlethwaite PA. Early expression of angiogenesis factors in acute myocardial ischemia and infarction. N Engl J Med 2000; 342(9):626-633.

Lemoli RM, Bertolini F, Cancedda R, De Luca M, Del Santo A, Ferrari G et al. Stem cell plasticity: time for a reappraisal? Haematologica 2005; 90(3):360-381.

Lenk K, Adams V, Lurz P, Erbs S, Linke A, Gielen S et al. Therapeutical potential of blood-derived progenitor cells in patients with peripheral arterial occlusive disease and critical limb ischaemia. Eur Heart J 2005; 26(18):1903-1909.

Li J, Yang C, Xia Y, Bertino A, Glaspy J, Roberts M et al. Thrombocytopenia caused by the development of antibodies to thrombopoietin. Blood 2001; 98(12):3241-3248.

Li TS, Ito H, Hayashi M, Furutani A, Matsuzaki M, Hamano K. Cellular expression of integrin-beta 1 is of critical importance for inducing therapeutic angiogenesis by cell implantation. Cardiovasc Res 2005; 65(1):64-72.

Lieber M, Smith B, Szakal A, Nelson-Rees W, Todaro G. A continuous tumor-cell line from a human lung carcinoma with properties of type II alveolar epithelial cells. Int J Cancer 1976; 17(1):62-70.

Lin Y, Weisdorf DJ, Solovey A, Hebbel RP. Origins of circulating endothelial cells and endothelial outgrowth from blood. J Clin Invest 2000; 105(1):71-77.

Lindemann A, Rumberger B. Vascular complications in patients treated with granulocyte colony-stimulating factor (G-CSF). Eur J Cancer 1993; 29A(16):2338-2339.

Llevadot J, Murasawa S, Kureishi Y, Uchida S, Masuda H, Kawamoto A et al. HMG-CoA reductase inhibitor mobilizes bone marrow—derived endothelial progenitor cells. J Clin Invest 2001; 108(3):399-405.

Loomans CJ, de Koning EJ, Staal FJ, Rookmaaker MB, Verseyden C, de Boer HC et al. Endothelial progenitor cell dysfunction: a novel concept in the pathogenesis of vascular complications of type 1 diabetes. Diabetes 2004; 53(1):195-199.

Lyden D, Hattori K, Dias S, Costa C, Blaikie P, Butros L et al. Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. Nat Med 2001; 7(11):1194-1201.

Makin AJ, Blann AD, Chung NA, Silverman SH, Lip GY. Assessment of endothelial damage in

atherosclerotic vascular disease by quantification of circulating endothelial cells. Relationship with von Willebrand factor and tissue factor. Eur Heart J 2004; 25(5):371-376.

Mancuso P, Burlini A, Pruneri G, Goldhirsch A, Martinelli G, Bertolini F. Resting and activated endothelial cells are increased in the peripheral blood of cancer patients. Blood 2001; 97(11):3658-3661.

Mannucci PM. Platelet von Willebrand factor in inherited and acquired bleeding disorders. Proc Natl Acad Sci U S A 1995; 92(7):2428-2432.

Marshak DRea. Stem Cell Biology. In: Marshak DRGRLaGD, editor. Stem Cell Biology. NY: ColdSpring Harbor Laboratory Press, 2001.

Martin-Rendon E, Watt SM. Stem cell plasticity. Br J Haematol 2003; 122(6):877-891.

Martinez-Estrada OM, Munoz-Santos Y, Julve J, Reina M, Vilaro S. Human adipose tissue as a source of Flk-1+ cells: new method of differentiation and expansion. Cardiovasc Res 2005; 65(2):328-333.

Martins RN, Chleboun JO, Sellers P, Sleigh M, Muir J. The role of PDGF-BB on the development of the collateral circulation after acute arterial occlusion. Growth Factors 1994; 10(4):299-306.

Massa M, Rosti V, Ferrario M, Campanelli R, Ramajoli I, Rosso R et al. Increased circulating hematopoietic and endothelial progenitor cells in the early phase of acute myocardial infarction. Blood 2005; 105(1):199-206.

Masuda H, Asahara T. Post-natal endothelial progenitor cells for neovascularization in tissue regeneration. Cardiovasc Res 2003; 58(2):390-398.

McNiece I, Jones R, Bearman SI, Cagnoni P, Nieto Y, Franklin W et al. Ex vivo expanded peripheral blood progenitor cells provide rapid neutrophil recovery after high-dose chemotherapy in patients with breast cancer. Blood 2000; 96(9):3001-3007.

McNiece I, Briddell R. Ex vivo expansion of hematopoietic progenitor cells and mature cells. Exp Hematol 2001; 29(1):3-11.

McNiece I. Ex vivo expansion of hematopoietic cells. Exp Hematol 2004; 32(5):409-410.

McNiece IK, Almeida-Porada G, Shpall EJ, Zanjani E. Ex vivo expanded cord blood cells provide rapid engraftment in fetal sheep but lack long-term engrafting potential. Exp Hematol 2002; 30(6):612-616.

Metcalf D, Moore AS, Shortman K. Adherence column and buoyant density separation of bone marrow stem cells and more differentiated cells. J Cell Physiol 1971; 78(3):441-450.

Mezey E, Key S, Vogelsang G, Szalayova I, Lange GD, Crain B. Transplanted bone marrow generates new neurons in human brains. Proc Natl Acad Sci U S A 2003; 100(3):1364-1369.

Minami E, Laflamme MA, Saffitz JE, Murry CE. Extracardiac progenitor cells repopulate most major cell types in the transplanted human heart. Circulation 2005; 112(19):2951-2958.

Mohle R, Bautz F, Rafii S, Moore MA, Brugger W, Kanz L. The chemokine receptor CXCR-4 is

expressed on CD34+ hematopoietic progenitors and leukemic cells and mediates transendothelial migration induced by stromal cell-derived factor-1. Blood 1998; 91(12):4523-4530.

Moldovan NI, Goldschmidt-Clermont PJ, Parker-Thornburg J, Shapiro SD, Kolattukudy PE. Contribution of monocytes/macrophages to compensatory neovascularization: the drilling of metalloelastase-positive tunnels in ischemic myocardium. Circ Res 2000; 87(5):378-384.

Montfort MJ, Olivares CR, Mulcahy JM, Fleming WH. Adult blood vessels restore host hematopoiesis following lethal irradiation. Exp Hematol 2002; 30(8):950-956.

Moore MA, Hattori K, Heissig B, Shieh JH, Dias S, Crystal RG et al. Mobilization of endothelial and hematopoietic stem and progenitor cells by adenovector-mediated elevation of serum levels of SDF-1, VEGF, and angiopoietin-1. Ann N Y Acad Sci 2001; 938:36-45.

Moore MA. Putting the neo into neoangiogenesis. J Clin Invest 2002; 109(3):313-315.

Morel F, Szilvassy SJ, Travis M, Chen B, Galy A. Primitive hematopoietic cells in murine bone marrow express the CD34 antigen. Blood 1996; 88(10):3774-3784.

Morel F, Galy A, Chen B, Szilvassy SJ. Equal distribution of competitive long-term repopulating stem cells in the CD34+ and. Exp Hematol 1998; 26(5):440-448.

Morimoto A, Sakata Y, Watanabe T, Murakami N. Leucocytosis induced in rabbits by intravenous or central injection of granulocyte colony stimulating factor. J Physiol 1990; 426:117-126.

Morishita R, Nakamura S, Hayashi S, Taniyama Y, Moriguchi A, Nagano T et al. Therapeutic angiogenesis induced by human recombinant hepatocyte growth factor in rabbit hind limb ischemia model as cytokine supplement therapy. Hypertension 1999; 33(6):1379-1384.

Muguruma Y, Reyes M, Nakamura Y, Sato T, Matsuzawa H, Miyatake H et al. In vivo and in vitro differentiation of myocytes from human bone marrow-derived multipotent progenitor cells. Exp Hematol 2003; 31(12):1323-1330.

Muller AM, Skrzynski C, Skipka G, Muller KM. Expression of von Willebrand factor by human pulmonary endothelial cells in vivo. Respiration 2002; 69(6):526-533.

Muller AM, Hermanns MI, Skrzynski C, Nesslinger M, Muller KM, Kirkpatrick CJ. Expression of the endothelial markers PECAM-1, vWf, and CD34 in vivo and in vitro. Exp Mol Pathol 2002; 72(3):221-229.

Murasawa S, Llevadot J, Silver M, Isner JM, Losordo DW, Asahara T. Constitutive human telomerase reverse transcriptase expression enhances regenerative properties of endothelial progenitor cells. Circulation 2002; 106(9):1133-1139.

Murasawa S, Asahara T. Endothelial progenitor cells for vasculogenesis. Physiology (Bethesda) 2005; 20:36-42.

Murohara T, Ikeda H, Duan J, Shintani S, Sasaki K, Eguchi H et al. Transplanted cord blood-derived endothelial precursor cells augment postnatal neovascularization. J Clin Invest 2000; 105(11):1527-1536.

Murohara T. Therapeutic vasculogenesis using human cord blood-derived endothelial progenitors. Trends Cardiovasc Med 2001; 11(8):303-307.

Murr, PDF. The development in vitro of the blood of the early chick embryo. Proc R Soc Lond B Biol Sci 1932; **111**:p. 497.

Nagasawa T, Nakajima T, Tachibana K, Iizasa H, Bleul CC, Yoshie O et al. Molecular cloning and characterization of a murine pre-B-cell growth-stimulating factor/stromal cell-derived factor 1 receptor, a murine homolog of the human immunodeficiency virus 1 entry coreceptor fusin. Proc Natl Acad Sci U S A 1996; 93(25):14726-14729.

Nagasawa T, Tachibana K, Kishimoto T. A novel CXC chemokine PBSF/SDF-1 and its receptor CXCR4: their functions in development, hematopoiesis and HIV infection. Semin Immunol 1998; 10(3):179-185.

Nagaya N, Kangawa K, Itoh T, Iwase T, Murakami S, Miyahara Y et al. Transplantation of mesenchymal stem cells improves cardiac function in a rat model of dilated cardiomyopathy. Circulation 2005; 112(8):1128-1135.

Nakagami H, Maeda K, Morishita R, Iguchi S, Nishikawa T, Takami Y et al. Novel autologous cell therapy in ischemic limb disease through growth factor secretion by cultured adipose tissue-derived stromal cells. Arterioscler Thromb Vasc Biol 2005; 25(12):2542-2547.

Nakano K, Adachi Y, Minamino K, Iwasaki M, Shigematsu A, Kiriyama N et al. Mechanisms underlying acceleration of blood flow recovery in ischemic limbs by macrophage colony-stimulating factor. Stem Cells 2006; 24(5):1274-1279.

Neel BG, Gu H, Pao L. The 'Shp'ing news: SH2 domain-containing tyrosine phosphatases in cell signaling. Trends Biochem Sci 2003; 28(6):284-293.

Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z. Vascular endothelial growth factor (VEGF) and its receptors. FASEB J 1999; 13(1):9-22.

Ng YY, van Kessel B, Lokhorst HM, Baert MR, van den Burg CM, Bloem AC et al. Gene-expression profiling of CD34+ cells from various hematopoietic stem-cell sources reveals functional differences in stem-cell activity. J Leukoc Biol 2004; 75(2):314-323.

Noga SJ. Graft engineering: the evolution of hematopoietic transplantation. J Hematother 1992; 1(1):3-17.

Nomura S, Dan K, Hotta T, Fujimura K, Ikeda Y. Effects of pegylated recombinant human megakaryocyte growth and development factor in patients with idiopathic thrombocytopenic purpura. Blood 2002; 100(2):728-730.

Nosal' R, Jancinova V, Danihelova E. Chloroquine: a multipotent inhibitor of human platelets in vitro. Thromb Res 2000; 98(5):411-421.

Oberlin E, Tavian M, Blazsek I, Peault B. Blood-forming potential of vascular endothelium in the human embryo. Development 2002; 129(17):4147-4157.

Okuda T, van Deursen J, Hiebert SW, Grosveld G, Downing JR. AML1, the target of multiple

chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. Cell 1996; 84(2):321-330.

Orkin SH. Diversification of haematopoietic stem cells to specific lineages. Nat Rev Genet 2000; 1(1):57-64.

Orkin SH, Zon LI. Hematopoiesis and stem cells: plasticity versus developmental heterogeneity. Nat Immunol 2002; 3(4):323-328.

Orlic D, Kajstura J, Chimenti S, Bodine DM, Leri A, Anversa P. Transplanted adult bone marrow cells repair myocardial infarcts in mice. Ann N Y Acad Sci 2001; 938:221-229.

Osawa M, Hanada K, Hamada H, Nakauchi H. Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science 1996; 273(5272):242-245.

Panepucci RA, Siufi JL, Silva WA, Jr., Proto-Siquiera R, Neder L, Orellana M et al. Comparison of gene expression of umbilical cord vein and bone marrow-derived mesenchymal stem cells. Stem Cells 2004; 22(7):1263-1278.

Papayannopoulou T. Current mechanistic scenarios in hematopoietic stem/progenitor cell mobilization. Blood 2004; 103(5):1580-1585.

Paquette RL, Dergham ST, Karpf E, Wang HJ, Slamon DJ, Souza L et al. Ex vivo expanded unselected peripheral blood: progenitor cells reduce posttransplantation neutropenia, thrombocytopenia, and anemia in patients with breast cancer. Blood 2000; 96(7):2385-2390.

Passier R, Mummery C. Origin and use of embryonic and adult stem cells in differentiation and tissue repair. Cardiovasc Res 2003; 58(2):324-335.

Pech-Amsellem MA, Myara I, Pico I, Maziere C, Maziere JC, Moatti N. Oxidative modifications of low-density lipoproteins (LDL) by the human endothelial cell line EA.hy 926. Experientia 1996; 52(3):234-238.

Pecora AL, Stiff P, Jennis A, Goldberg S, Rosenbluth R, Price P et al. Prompt and durable engraftment in two older adult patients with high risk chronic myelogenous leukemia (CML) using ex vivo expanded and unmanipulated unrelated umbilical cord blood. Bone Marrow Transplant 2000; 25(7):797-799.

Peichev M, Naiyer AJ, Pereira D, Zhu Z, Lane WJ, Williams M et al. Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. Blood 2000; 95(3):952-958.

Peled A, Petit I, Kollet O, Magid M, Ponomaryov T, Byk T et al. Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. Science 1999; 283(5403):845-848.

Peled T, Mandel J, Goudsmid RN, Landor C, Hasson N, Harati D et al. Pre-clinical development of cord blood-derived progenitor cell graft expanded ex vivo with cytokines and the polyamine copper chelator tetraethylenepentamine. Cytotherapy 2004; 6(4):344-355.

Pelosi E, Valtieri M, Coppola S, Botta R, Gabbianelli M, Lulli V et al. Identification of the

hemangioblast in postnatal life. Blood 2002; 100(9):3203-3208.

Pera MF, Filipczyk AA, Hawes SM, Laslett AL. Isolation, characterization, and differentiation of human embryonic stem cells. Methods Enzymol 2003; 365:429-446.

Perin EC, Geng YJ, Willerson JT. Adult stem cell therapy in perspective. Circulation 2003; 107(7):935-938.

Pesce M, Orlandi A, Iachininoto MG, Straino S, Torella AR, Rizzuti V et al. Myoendothelial differentiation of human umbilical cord blood-derived stem cells in ischemic limb tissues. Circ Res 2003; 93(5):e51-e62.

Pillarisetti K, Gupta SK. Cloning and relative expression analysis of rat stromal cell derived factor-1 (SDF-1)1: SDF-1 alpha mRNA is selectively induced in rat model of myocardial infarction. Inflammation 2001; 25(5):293-300.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD et al. Multilineage potential of adult human mesenchymal stem cells. Science 1999; 284(5411):143-147.

Pittenger MF, Martin BJ. Mesenchymal stem cells and their potential as cardiac therapeutics. Circ Res 2004; 95(1):9-20.

Pizzo PA. Granulocytopenia and cancer therapy. Past problems, current solutions, future challenges. Cancer 1984; 54(11 Suppl):2649-2661.

Planat-Benard V, Silvestre JS, Cousin B, Andre M, Nibbelink M, Tamarat R et al. Plasticity of human adipose lineage cells toward endothelial cells: physiological and therapeutic perspectives. Circulation 2004; 109(5):656-663.

Post MJ. Angiogenesis: initiation and maintenance. Ann N Y Acad Sci 2002; 961:249-250.

Powell TM, Paul JD, Hill JM, Thompson M, Benjamin M, Rodrigo M et al. Granulocyte colony-stimulating factor mobilizes functional endothelial progenitor cells in patients with coronary artery disease. Arterioscler Thromb Vasc Biol 2005; 25(2):296-301.

Price TH. Granulocyte colony-stimulating factor-mobilized granulocyte concentrate transfusions. Curr Opin Hematol 1998; 5(6):391-395.

Prince HM, Simmons PJ, Whitty G, Wall DP, Barber L, Toner GC et al. Improved haematopoietic recovery following transplantation with ex vivo-expanded mobilized blood cells. Br J Haematol 2004; 126(4):536-545.

Quesenberry PJ, Colvin GA, Lambert JF. The chiaroscuro stem cell: a unified stem cell theory. Blood 2002; 100(13):4266-4271.

Quirici N, Soligo D, Caneva L, Servida F, Bossolasco P, Deliliers GL. Differentiation and expansion of endothelial cells from human bone marrow CD133(+) cells. Br J Haematol 2001; 115(1):186-194.

Rafii S, Lyden D. Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration. Nat Med 2003; 9(6):702-712.

Rasmusson I, Ringden O, Sundberg B, Le Blanc K. Mesenchymal stem cells inhibit the formation

of cytotoxic T lymphocytes, but not activated cytotoxic T lymphocytes or natural killer cells. Transplantation 2003; 76(8):1208-1213.

Ratajczak MZ, Kucia M, Majka M, Reca R, Ratajczak J. Heterogeneous populations of bone marrow stem cells—are we spotting on the same cells from the different angles? Folia Histochem Cytobiol 2004; 42(3):139-146.

Rehman J, Li J, Orschell CM, March KL. Peripheral blood "endothelial progenitor cells" are derived from monocyte/macrophages and secrete angiogenic growth factors. Circulation 2003; 107(8):1164-1169.

Rehman J, Li J, Parvathaneni L, Karlsson G, Panchal VR, Temm CJ et al. Exercise acutely increases circulating endothelial progenitor cells and monocyte-/macrophage-derived angiogenic cells. J Am Coll Cardiol 2004; 43(12):2314-2318.

Reichle A, Zaiss M, Rothe G, Schmitz G, Andreesen R. Autologous tandem transplantation: almost complete reduction of neutropenic fever following the second transplantation by ex vivo expanded autologous myeloid postprogenitor cells. Bone Marrow Transplant 2003; 32(3):299-305.

Reiffers J, Cailliot C, Dazey B, Attal M, Caraux J, Boiron JM. Abrogation of post-myeloablative chemotherapy neutropenia by ex-vivo expanded autologous CD34-positive cells. Lancet 1999; 354(9184):1092-1093.

Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol 2000; 18(4):399-404.

Reyes M, Verfaillie CM. Characterization of multipotent adult progenitor cells, a subpopulation of mesenchymal stem cells. Ann N Y Acad Sci 2001; 938:231-233.

Reyes M, Dudek A, Jahagirdar B, Koodie L, Marker PH, Verfaillie CM. Origin of endothelial progenitors in human postnatal bone marrow. J Clin Invest 2002; 109(3):337-346.

Ricousse-Roussanne S, Barateau V, Contreres JO, Boval B, Kraus-Berthier L, Tobelem G. Ex vivo differentiated endothelial and smooth muscle cells from human cord blood progenitors home to the angiogenic tumor vasculature. Cardiovasc Res 2004; 62(1):176-184.

Ringden O, Sundberg B, Lonnqvist B, Tollemar J, Gahrton G, Nilsson B. Allogeneic bone marrow transplantation for leukemia: factors of importance for long-term survival and relapse. Bone Marrow Transplant 1988; 3(4):281-290.

Risau W, Flamme I. Vasculogenesis. Annu Rev Cell Dev Biol 1995; 11:73-91.

Robb L, Lyons I, Li R, Hartley L, Kontgen F, Harvey RP et al. Absence of yolk sac hematopoiesis from mice with a targeted disruption of the scl gene. Proc Natl Acad Sci U S A 1995; 92(15):7075-7079.

Robinson S, Niu T, de Lima M, Ng J, Yang H, McMannis J et al. Ex vivo expansion of umbilical cord blood. Cytotherapy 2005; 7(3):243-250.

Rohde E, Malischnik C, Thaler D, Maierhofer T, Linkesch W, Lanzer G et al. Blood monocytes

mimic endothelial progenitor cells. Stem Cells 2006; 24(2):357-367.

Romagnani P, Annunziato F, Liotta F, Lazzeri E, Mazzinghi B, Frosali F et al. CD14+CD34low cells with stem cell phenotypic and functional features are the major source of circulating endothelial progenitors. Circ Res 2005; 97(4):314-322.

Rookmaaker MB, Vergeer M, van Zonneveld AJ, Rabelink TJ, Verhaar MC. Endothelial progenitor cells: mainly derived from the monocyte/macrophage-containing. Circulation 2003; 108(21):e150.

Rubinstein P, Carrier C, Scaradavou A, Kurtzberg J, Adamson J, Migliaccio AR et al. Outcomes among 562 recipients of placental-blood transplants from unrelated donors. N Engl J Med 1998; 339(22):1565-1577.

Sabin F. Studies on the origin of blood vessels and of red blood corpuscles as seen in the living blastoderm of chicks during the second day of incubation. Contrib Embryol Carnegie Institute Pub 1920; 214:p. 9.

Salven P, Lymboussaki A, Heikkila P, Jaaskela-Saari H, Enholm B, Aase K et al. Vascular endothelial growth factors VEGF-B and VEGF-C are expressed in human tumors. Am J Pathol 1998; 153(1):103-108.

Sata M, Takahashi A, Tanaka K, Washida M, Ishizaka N, Ako J et al. Mouse genetic evidence that tranilast reduces smooth muscle cell hyperplasia via a p21(WAF1)-dependent pathway. Arterioscler Thromb Vasc Biol 2002; 22(8):1305-1309.

Schachinger V, Assmus B, Britten MB, Honold J, Lehmann R, Teupe C et al. Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction: final one-year results of the TOPCARE-AMI Trial. J Am Coll Cardiol 2004; 44(8):1690-1699.

Schachinger V, Assmus B, Britten MB, Honold J, Lehmann R, Teupe C et al. Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction: final one-year results of the TOPCARE-AMI Trial. J Am Coll Cardiol 2004; 44(8):1690-1699.

Schatteman GC, Hanlon HD, Jiao C, Dodds SG, Christy BA. Blood-derived angioblasts accelerate blood-flow restoration in diabetic mice. J Clin Invest 2000; 106(4):571-578.

Scheding S, Meister B, Buhring HJ, Baum CM, Mc Kearn JP, Bock T et al. Effective ex vivo generation of granulopoietic postprogenitor cells from mobilized peripheral blood CD34(+) cells. Exp Hematol 2000; 28(4):460-470.

Scheding S, Bergmannn M, Rathke G, Vogel W, Brugger W, Kanz L. Additional transplantation of ex vivo generated megakaryocytic cells after high-dose chemotherapy. Haematologica 2004; 89(5):630-631.

Schmeisser A, Garlichs CD, Zhang H, Eskafi S, Graffy C, Ludwig J et al. Monocytes coexpress endothelial and macrophagocytic lineage markers and form cord-like structures in Matrigel under angiogenic conditions. Cardiovasc Res 2001; 49(3):671-680.

Serruys PW, de Jaegere P, Kiemeneij F, Macaya C, Rutsch W, Heyndrickx G et al. A comparison

of balloon-expandable-stent implantation with balloon angioplasty in patients with coronary artery disease. Benestent Study Group. N Engl J Med 1994; 331(8):489-495.

Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML et al. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature 1995; 376(6535):62-66.

Shi Q (a), Rafii S, Wu MH, Wijelath ES, Yu C, Ishida A et al. Evidence for circulating bone marrow-derived endothelial cells. Blood 1998; 92(2):362-367.

Shi Q (b), Wu MH, Onuki Y, Kouchi Y, Ghali R, Wechezak AR et al. The effect of flow shear stress on endothelialization of impervious Dacron grafts from circulating cells in the arterial and venous systems of the same dog. Ann Vasc Surg 1998; 12(4):341-348.

Shimizu K, Sugiyama S, Aikawa M, Fukumoto Y, Rabkin E, Libby P et al. Host bone-marrow cells are a source of donor intimal smooth- muscle-like cells in murine aortic transplant arteriopathy. Nat Med 2001; 7(6):738-741.

Shimizu T, Yamato M, Isoi Y, Akutsu T, Setomaru T, Abe K et al. Fabrication of pulsatile cardiac tissue grafts using a novel 3-dimensional cell sheet manipulation technique and temperature-responsive cell culture surfaces. Circ Res 2002; 90(3):e40.

Shintani S, Murohara T, Ikeda H, Ueno T, Sasaki K, Duan J et al. Augmentation of postnatal neovascularization with autologous bone marrow transplantation. Circulation 2001; 103(6):897-903.

Shivdasani RA, Mayer EL, Orkin SH. Absence of blood formation in mice lacking the T-cell leukaemia oncoprotein tal-1/SCL. Nature 1995; 373(6513):432-434.

Shmelkov SV, St Clair R, Lyden D, Rafii S. AC133/CD133/Prominin-1. Int J Biochem Cell Biol 2005; 37(4):715-719.

Shpall EJ, Quinones R, Giller R, Zeng C, Baron AE, Jones RB et al. Transplantation of ex vivo expanded cord blood. Biol Blood Marrow Transplant 2002; 8(7):368-376.

Siena S, Schiavo R, Pedrazzoli P, Carlo-Stella C. Therapeutic relevance of CD34 cell dose in blood cell transplantation for cancer therapy. J Clin Oncol 2000; 18(6):1360-1377.

Silva GV, Litovsky S, Assad JA, Sousa AL, Martin BJ, Vela D et al. Mesenchymal stem cells differentiate into an endothelial phenotype, enhance vascular density, and improve heart function in a canine chronic ischemia model. Circulation 2005; 111(2):150-156.

Stacker SA, Achen MG, Jussila L, Baldwin ME, Alitalo K. Lymphangiogenesis and cancer metastasis. Nat Rev Cancer 2002; 2(8):573-583.

Stainier DY, Weinstein BM, Detrich HW, III, Zon LI, Fishman MC. Cloche, an early acting zebrafish gene, is required by both the endothelial and hematopoietic lineages. Development 1995; 121(10):3141-3150.

Stamm C, Westphal B, Kleine HD, Petzsch M, Kittner C, Klinge H et al. Autologous bone-marrow stem-cell transplantation for myocardial regeneration. Lancet 2003; 361(9351):45-46.

Steiner S, Niessner A, Ziegler S, Richter B, Seidinger D, Pleiner J et al. Endurance training increases the number of endothelial progenitor cells in patients with cardiovascular risk and coronary artery disease. Atherosclerosis 2005; 181(2):305-310.

Stem Cells. Stem cells: Scientific Progress and Future and research directions. Department of Health and Human Services2001. Eletronic citation.

Strauer BE, Brehm M, Zeus T, Kostering M, Hernandez A, Sorg RV et al. Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. Circulation 2002; 106(15):1913-1918.

Strauer BE, Kornowski R. Stem cell therapy in perspective. Circulation 2003; 107(7):929-934.

Strauss RG. Therapeutic granulocyte transfusions in 1993. Blood 1993; 81(7):1675-1678.

Strauss RG. Rebirth of granulocyte transfusions: should it involve pediatric oncology and transplant patients? J Pediatr Hematol Oncol 1999; 21(6):475-478.

Strehlow K, Werner N, Berweiler J, Link A, Dirnagl U, Priller J et al. Estrogen increases bone marrow-derived endothelial progenitor cell production and diminishes neointima formation. Circulation 2003; 107(24):3059-3065.

Stump MM, Jordan GL,Jr.,Debakey ME, Halpert B.Endothelium grown from circulating blood on isolated intravascular dacron hub.Am J Pathol 1963; 43:361-367.

Su EJ, Cioffi CL, Stefansson S, Mittereder N, Garay M, Hreniuk D et al. Gene therapy vector-mediated expression of insulin-like growth factors protects cardiomyocytes from apoptosis and enhances neovascularization. Am J Physiol Heart Circ Physiol 2003; 284(4):H1429-H1440.

Sutherland DR, Anderson L, Keeney M, Nayar R, Chin-Yee I. The ISHAGE guidelines for CD34+ cell determination by flow cytometry. International Society of Hematotherapy and Graft Engineering. J Hematother 1996; 5(3):213-226.

Suzuki T, Nishida M, Futami S, Fukino K, Amaki T, Aizawa K et al. Neoendothelialization after peripheral blood stem cell transplantation in humans: a case report of a Tokaimura nuclear accident victim. Cardiovasc Res 2003; 58(2):487-492.

Takahashi T, Kalka C, Masuda H, Chen D, Silver M, Kearney M et al. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. Nat Med 1999; 5(4):434-438.

Takahashi T, Lord B, Schulze PC, Fryer RM, Sarang SS, Gullans SR et al. Ascorbic acid enhances differentiation of embryonic stem cells into cardiac myocytes. Circulation 2003; 107(14):1912-1916.

Takakura N, Watanabe T, Suenobu S, Yamada Y, Noda T, Ito Y et al. A role for hematopoietic stem cells in promoting angiogenesis. Cell 2000; 102(2):199-209.

Tamarat R, Silvestre JS, Ricousse-Roussanne S, Barateau V, Lecomte-Raclet L, Clergue M et al. Impairment in ischemia-induced neovascularization in diabetes: bone marrow mononuclear cell dysfunction and therapeutic potential of placenta growth factor

treatment. Am J Pathol 2004; 164(2):457-466.

Tang YL, Zhao Q, Zhang YC, Cheng L, Liu M, Shi J et al. Autologous mesenchymal stem cell transplantation induce VEGF and neovascularization in ischemic myocardium. Regul Pept 2004; 117(1):3-10.

Tateishi-Yuyama E, Matsubara H, Murohara T, Ikeda U, Shintani S, Masaki H et al. Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: a pilot study and a randomised controlled trial. Lancet 2002; 360(9331):427-435.

Tavian M, Coulombel L, Luton D, Clemente HS, Dieterlen-Lievre F, Peault B. Aorta-associated CD34+ hematopoietic cells in the early human embryo. Blood 1996; 87(1):67-72.

Tepper OM, Galiano RD, Capla JM, Kalka C, Gagne PJ, Jacobowitz GR et al. Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures. Circulation 2002; 106(22):2781-2786.

Theise ND (a), Badve S, Saxena R, Henegariu O, Sell S, Crawford JM et al. Derivation of hepatocytes from bone marrow cells in mice after radiation-induced myeloablation. Hepatology 2000; 31(1):235-240.

Theise ND (b), Nimmakayalu M, Gardner R, Illei PB, Morgan G, Teperman L et al. Liver from bone marrow in humans. Hepatology 2000; 32(1):11-16.

Thomas J, Liu F, Link DC. Mechanisms of mobilization of hematopoietic progenitors with granulocyte colony-stimulating factor. Curr Opin Hematol 2002; 9(3):183-189.

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS et al. Embryonic stem cell lines derived from human blastocysts. Science 1998; 282(5391):1145-1147.

To LB, Roberts MM, Haylock DN, Dyson PG, Branford AL, Thorp D et al. Comparison of haematological recovery times and supportive care requirements of autologous recovery phase peripheral blood stem cell transplants, autologous bone marrow transplants and allogeneic bone marrow transplants. Bone Marrow Transplant 1992; 9(4):277-284.

Tonks NK, Neel BG. From form to function: signaling by protein tyrosine phosphatases. Cell 1996; 87(3):365-368.

Tornebohm E, Lockner D, Paul C. A retrospective analysis of bleeding complications in 438 patients with acute leukaemia during the years 1972-1991. Eur J Haematol 1993; 50(3):160-167.

Tsai RY, McKay RD. Cell contact regulates fate choice by cortical stem cells. J Neurosci 2000; 20(10):3725-3735.

Tse HF, Kwong YL, Chan JK, Lo G, Ho CL, Lau CP. Angiogenesis in ischaemic myocardium by intramyocardial autologous bone marrow mononuclear cell implantation. Lancet 2003; 361(9351):47-49.

Tse WT, Pendleton JD, Beyer WM, Egalka MC, Guinan EC. Suppression of allogeneic T-cell

proliferation by human marrow stromal cells: implications in transplantation. Transplantation 2003; 75(3):389-397.

Turk B, Turk D, Turk V. Lysosomal cysteine proteases: more than scavengers. Biochim Biophys Acta 2000; 1477(1-2):98-111.

Tutschka PJ. Diminishing morbidity and mortality of bone marrow transplantation. Vox Sang 1986; 51 Suppl 2:87-94.

Uchida N, Buck DW, He D, Reitsma MJ, Masek M, Phan TV et al. Direct isolation of human central nervous system stem cells. Proc Natl Acad Sci U S A 2000; 97(26):14720-14725.

Uher F, Hajdu M, Vas V. Self-renewal and differentiation of hematopoietic stem cells: a molecular approach (a review). Acta Microbiol Immunol Hung 2003; 50(1):3-21.

Urbich C. Isolation culture, and differentiation of circulating endothelial precursor cells. 2005. Personal Communication

Urbich C (a), Aicher A, Heeschen C, Dernbach E, Hofmann WK, Zeiher AM et al. Soluble factors released by endothelial progenitor cells promote migration of endothelial cells and cardiac resident progenitor cells. J Mol Cell Cardiol 2005; 39(5):733-742.

Urbich C (b), Heeschen C, Aicher A, Sasaki K, Bruhl T, Farhadi MR et al. Cathepsin L is required for endothelial progenitor cell-induced neovascularization. Nat Med 2005; 11(2):206-213.

Urbich C, Heeschen C, Aicher A, Dernbach E, Zeiher AM, Dimmeler S. Relevance of monocytic features for neovascularization capacity of circulating endothelial progenitor cells. Circulation 2003; 108(20):2511-2516.

Urbich C, Dimmeler S. Endothelial progenitor cells functional characterization. Trends Cardiovasc Med 2004; 14(8):318-322.

Vajanto I, Rissanen TT, Rutanen J, Hiltunen MO, Tuomisto TT, Arve K et al. Evaluation of angiogenesis and side effects in ischemic rabbit hindlimbs after intramuscular injection of adenoviral vectors encoding VEGF and LacZ. J Gene Med 2002; 4(4):371-380.

Vajkoczy P, Blum S, Lamparter M, Mailhammer R, Erber R, Engelhardt B et al. Multistep nature of microvascular recruitment of ex vivo-expanded embryonic endothelial progenitor cells during tumor angiogenesis. J Exp Med 2003; 197(12):1755-1765.

van de WL. What has universal leucodepletion given us: evidence from clinical trials? Vox Sang 2004; 87 Suppl 2:139-142.

Vasa M, Fichtlscherer S, Adler K, Aicher A, Martin H, Zeiher AM et al. Increase in circulating endothelial progenitor cells by statin therapy in patients with stable coronary artery disease. Circulation 2001; 103(24):2885-2890.

Vassilopoulos G, Wang PR, Russell DW. Transplanted bone marrow regenerates liver by cell fusion. Nature 2003; 422(6934):901-904.

Verfaillie C (a), Pera MF, Lansdorp PM. Stem cells: hype and reality. Hematology Am Soc Hematol Educ Program 2002;369-391.

Verfaillie CM (b). Hematopoietic stem cells for transplantation. Nat Immunol 2002; 3(4):314-317.

Volin MV, Joseph L, Shockley MS, Davies PF. Chemokine receptor CXCR4 expression in endothelium. Biochem Biophys Res Commun 1998; 242(1):46-53.

Vulliet PR, Greeley M, Halloran SM, MacDonald KA, Kittleson MD. Intra-coronary arterial injection of mesenchymal stromal cells and microinfarction in dogs. Lancet 2004; 363(9411):783-784.

Walenta K, Friedrich EB, Sehnert F, Werner N, Nickenig G. In vitro differentiation characteristics of cultured human mononuclear cells-implications for endothelial progenitor cell biology. Biochem Biophys Res Commun 2005; 333(2):476-482.

Wall DM, Prince HM. Regulation of cellular therapies: the Australian perspective. Cytotherapy 2003; 5(4):284-288.

Walter DH, Rittig K, Bahlmann FH, Kirchmair R, Silver M, Murayama T et al. Statin therapy accelerates reendothelialization: a novel effect involving mobilization and incorporation of bone marrow-derived endothelial progenitor cells. Circulation 2002; 105(25):3017-3024.

Wang Q, Stacy T, Miller JD, Lewis AF, Gu TL, Huang X et al. The CBFbeta subunit is essential for CBFalpha2 (AML1) function in vivo. Cell 1996; 87(4):697-708.

Wang X, Willenbring H, Akkari Y, Torimaru Y, Foster M, Al Dhalimy M et al. Cell fusion is the principal source of bone-marrow-derived hepatocytes. Nature 2003; 422(6934):897-901.

Weinmann P, Scharffetter-Kochanek K, Forlow SB, Peters T, Walzog B. A role for apoptosis in the control of neutrophil homeostasis in the circulation: insights from CD18-deficient mice. Blood 2003; 101(2):739-746.

Werner N, Junk S, Laufs U, Link A, Walenta K, Bohm M et al. Intravenous transfusion of endothelial progenitor cells reduces neointima formation after vascular injury. Circ Res 2003; 93(2):e17-e24.

Witmer AN, Vrensen GF, Van Noorden CJ, Schlingemann RO. Vascular endothelial growth factors and angiogenesis in eye disease. Prog Retin Eye Res 2003; 22(1):1-29.

Wollert KC, Drexler H. Cell therapy for acute myocardial infarction: where are we heading? Nat Clin Pract Cardiovasc Med 2004; 1(2):61.

Wright HP, Glacometti NJ. Circulating endothelial cells and arterial endothelial mitosis in anaphylactic shock. Br J Exp Pathol 1972; 53(1):1-4.

Wurmser AE, Nakashima K, Summers RG, Toni N, D'Amour KA, Lie DC et al. Cell fusion-independent differentiation of neural stem cells to the endothelial lineage. Nature 2004; 430(6997):350-356.

Yamada KM, Araki M. Tumor suppressor PTEN: modulator of cell signaling, growth, migration and apoptosis. J Cell Sci 2001; 114(Pt 13):2375-2382.

Yamaguchi J, Kusano KF, Masuo O, Kawamoto A, Silver M, Murasawa S et al. Stromal cell-

derived factor-1 effects on ex vivo expanded endothelial progenitor cell recruitment for ischemic neovascularization. Circulation 2003; 107(9):1322-1328.

Yarnal JR, Hollis TM. Circulating endothelial cells in experimental hypertension: possible thrombogenic role of histidine decarboxylase. J Am Osteopath Assoc 1974; 73(5):402-404.

Yeghen T, Devereux S. Granulocyte transfusion: a review. Vox Sang 2001; 81(2):87-92.

Yin AH, Miraglia S, Zanjani ED, Almeida-Porada G, Ogawa M, Leary AG et al. AC133, a novel marker for human hematopoietic stem and progenitor cells. Blood 1997; 90(12):5002-5012.

Yoder MC, Hiatt K. Engraftment of embryonic hematopoietic cells in conditioned newborn recipients. Blood 1997; 89(6):2176-2183.

Yoon YS, Park JS, Tkebuchava T, Luedeman C, Losordo DW. Unexpected severe calcification after transplantation of bone marrow cells in acute myocardial infarction. Circulation 2004; 109(25):3154-3157.

Yoshioka T, Takahashi M, Shiba Y, Suzuki C, Morimoto H, Izawa A et al. Granulocyte colony-stimulating factor (G-CSF) accelerates reendothelialization and reduces neointimal formation after vascular injury in mice. Cardiovasc Res 2006; 70(1):61-69.

Yun I, Cho ES, Jang HO, Kim UK, Choi CH, Chung IK et al. Amphiphilic effects of local anesthetics on rotational mobility in neuronal and model membranes. Biochim Biophys Acta 2002; 1564(1):123-132.

Zammaretti P, Zisch AH. Adult 'endothelial progenitor cells'. Renewing vasculature. Int J Biochem Cell Biol 2005; 37(3):493-503.

Zhang H, Vakil V, Braunstein M, Smith EL, Maroney J, Chen L et al. Circulating endothelial progenitor cells in multiple myeloma: implications and significance. Blood 2005; 105(8):3286-3294.

Zhang J, Niu C, Ye L, Huang H, He X, Tong WG et al. Identification of the haematopoietic stem cell niche and control of the niche size. Nature 2003; 425(6960):836-841.

Zhang SJ, Zhang H, Wei YJ, Su WJ, Liao ZK, Hou M et al. Adult endothelial progenitor cells from human peripheral blood maintain monocyte/macrophage function throughout in vitro culture. Cell Res 2006; 16(6):577-584.

Zhao Y, Glesne D, Huberman E. A human peripheral blood monocyte-derived subset acts as pluripotent stem cells. Proc Natl Acad Sci U S A 2003; 100(5):2426-2431.

Zimmerman TM, Lee WJ, Bender JG, Schilling M, Smith SL, Van Epps DE et al. Clinical impact of ex vivo differentiated myeloid precursors after high-dose chemotherapy and peripheral blood progenitor cell rescue. Bone Marrow Transplant 2000; 26(5):505-510.

Zimmet JM, Hare JM. Emerging role for bone marrow derived mesenchymal stem cells in myocardial regenerative therapy. Basic Res Cardiol 2005; 100(6):471-481.

Zipori D. The stem state: plasticity is essential, whereas self-renewal and hierarchy are optional. Stem Cells 2005; 23(6):719-726.

Zisch AH. Tissue engineering of angiogenesis with autologous endothelial progenitor cells. Curr Opin Biotechnol 2004; 15(5):424-429.

Zon LI. Developmental biology of hematopoiesis. Blood 1995; 86(8):2876-2891.

Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H et al. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 2002; 13(12):4279-4295.